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(54) Title: RECOMBINANT HOSTS SUITABLE FOR SIMULTANEOUS SACCHARIFICATION AND FERMENTATION

(57) Abstract: The invention provides recombinant host cells containing at least one heterologous polynucleotide encoding a polysaccharase under the transcriptional control of a surrogate promoter capable of increasing the expression of the polysaccharase. In addition, the invention further provides such hosts with genes encoding secretory protein/s to facilitate the secretion of the expressed polysaccharase. Preferred hosts of the invention are ethanologenic and capable of carrying out simultaneous saccharification fermentation resulting in the production of ethanol from complex cellulose substrates.

RECOMBINANT HOSTS SUITABLE FOR SIMULTANEOUS SACCHARIFICATION AND FERMENTATION

Related Information

This application claims priority to U.S. provisional Application No. 60/136,376, entitled "RECOMBINANT HOSTS SUITABLE FOR SIMULTANEOUS SACCHARIFICATION AND FERMENTATION," filed on may 26, 1999, incorporated herein in its entirety by this reference. The contents of all patents, patent applications, and references cited throughout this specification are hereby incorporated by reference in their entireties.

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Background of the Invention

Many environmental and societal benefits would result from the replacement of petroleum-based automotive fuels with renewable fuels obtained from plant materials (Lynd *et al.*, (1991) *Science* 251:1318-1323; Olson *et al.*, (1996) *Enzyme Microb. Technol.* 18:1-17; Wyman *et al.*, (1995) *Amer. Chem. Soc. Symp.* 618:272-290). Each year, the United States burns over 120 billion gallons of automotive fuel, roughly equivalent to the total amount of imported petroleum. The development of ethanol as a renewable alternative fuel has the potential to eliminate United States dependence on imported oil, improve the environment, and provide new employment (Sheehan, (1994) ACS Symposium Series No. 566, ACS Press, pp 1-53).

In theory, the solution to the problem of imported oil for automotive fuel appears quite simple. Rather than using petroleum, a finite resource, the ethanol can be produced efficiently by the fermentation of plant material, a renewable resource. Indeed, Brazil has demonstrated the feasibility of producing ethanol and the use of ethanol as a primary automotive fuel for more than 20 years. Similarly, the United States produces over 1.2 billion gallons of fuel ethanol each year. Currently, fuel

ethanol is produced from corn starch or cane syrup utilizing either *Saccharomyces cerevisiae* or *Zymomonas mobilis* (*Z. mobilis*). However, neither of these sugar sources can supply the volumes needed to realize a replacement of petroleum-based automotive fuels. In addition, both cane sugar and corn starch are relatively expensive starting materials which have competing uses as food products.

Moreover, these sugar substrates represent only a fraction of the total carbohydrates in plants. Indeed, the majority of the carbohydrates in plants is in the form of lignocellulose, a complex structural polymer containing cellulose, hemicellulose, pectin, and lignin. Lignocellulose is found in, for example, the stems, leaves, hulls, husks, and cobs of plants. Hydrolysis of these polymers releases a mixture of neutral sugars including glucose, xylose, mannose, galactose, and arabinose. No known natural organism can rapidly and efficiently metabolize all these sugars into ethanol.

Nonetheless, in an effort to exploit this substrate source, the Gulf Oil Company developed a method for the production of ethanol from cellulose using a yeast-based process termed simultaneous saccharification and fermentation (SSF) (Gauss *et al.* (1976) U.S.P.N. 3,990,944). Fungal cellulase preparations and yeasts were added to a slurry of the cellulosic substrate in a single vessel. Ethanol was produced concurrently during cellulose hydrolysis. However, Gulf's SSF process has some shortcomings. For example, fungal cellulases have been considered, thus far, to be too expensive for use in large scale bioethanol processes (Himmel *et al.*, (1997) Amer. Chem. Soc. pp. 2-45; Ingram *et al.*, (1987) *Appl. Environ. Microbiol.* 53:2420-2425; Okamoto *et al.*, (1994) *Appl. Microbiol. Biotechnol.* 42:563-568; Philippidis, G., (1994) Amer. Chem. Soc. pp. 188-217; Saito *et al.*, (1990) *J. Ferment. Bioeng.* 69:282-286; Sheehan, J., (1994) Amer. Chem. Soc. pp 1-52; Su *et al.*, (1993) *Biotechnol. Lett.* 15:979-984).

Summary of the Invention

The development of inexpensive enzymatic methods for cellulose hydrolysis has great potential for improving the efficiency of substrate utilization and the economics of the saccharification and fermentation process. Accordingly, developing a biocatalyst which can be used for the efficient depolymerization of a complex cellulosic substrate

and subsequent rapid fermentation of the substrate into ethanol, would be of great benefit.

The present invention provides a recombinant host cell engineered for increased expression and secretion of a polysaccharase suitable for depolymerizing complex carbohydrates. Specifically exemplified are two recombinant enteric bacteria, *Escherichia coli* and *Klebsiella oxytoca*, which express a polysaccharase at high levels under the transcriptional control of a surrogate promoter. The invention provides for the further modification of these hosts to include a secretory protein/s which allows for the increased production of polysaccharase in cell. In a preferred embodiment, the polysaccharase is produced in either increased amounts, with increased activity, or a combination thereof. In a preferred embodiment, the invention provides for the further modification of these hosts to include exogenous ethanologenic genes derived from an efficient ethanol producer, such as *Zymomonas mobilis*. Accordingly, these hosts are capable of expressing high levels of proteins that may be used alone or in combination with other enzymes or recombinant hosts for the efficient production of ethanol from complex carbohydrates.

More particularly, in a first aspect, the present invention features a recombinant host cell having increased production of a polysaccharase. The host cell of this aspect contains a heterologous polynucleotide segment containing a sequence that encodes a polysaccharase where the sequence is under the transcriptional control of a surrogate promoter and this promoter is capable of causing increased production of the polysaccharase. In addition, this aspect features a host cell that also contains a second heterologous polynucleotide segment containing a sequence that encodes a secretory polypeptide. The expression of the first and second heterologous polynucleotide segments results in the increased production of polysaccharase amounts, activity, or a combination thereof, by the recombinant host cell.

In a preferred embodiment, the polysaccharase polypeptide is secreted.

In another embodiment, the host cell is a bacterial cell, preferably Gram-negative, facultatively anaerobic, and from the family Enterobacteriaceae. In another related embodiment, the recombinant host cell is of the genus *Escherichia* or *Klebsiella* and, preferably, is the strain *E. coli* B, *E. coli* DH5α, *E. coli* KO4 (ATCC 55123), *E.*

coli KO11 (ATCC 55124), *E. coli* KO12 (ATCC 55125), *E. coli* LY01, *K. oxytoca* M5A1, or *K. oxytoca* P2 (ATCC 55307).

In another embodiment, the recombinant host contains a polynucleotide segment that encodes a polysaccharase that is a glucanase, endoglucanase, exoglucanase, cellobiohydrolase, β-glucosidase, endo-1,4-β-xylanase, α-xylosidase, α-glucuronidase, α-L-arabinofuranosidase, acetylesterase, acetylxylanesterase, α-amylase, β-amylase, glucoamylase, pullulanase, β-glucanase, hemicellulase, arabinosidase, mannanase, pectin hydrolase, pectate lyase, or may be a combination of these polysaccharases. In a related embodiment, the polysaccharase is preferably a glucanase, more preferably an expression product of a *celZ* gene, and most preferably, derived from *Erwinia chrysanthemi*.

In yet another embodiment, the recombinant host cell expresses a secretory polypeptide encoded by a *pul* or *out* gene preferably derived from a bacterial cell selected from the family Enterobacteriaceae and more preferably, from *K. oxytoca, E. carotovora, E. carotovora* subspecies *carotovora, E. carotovora* subspecies *atroseptica,* or *E. chrysanthemi*.

In a further embodiment, the surrogate promoter for driving gene expression in the recombinant host cell is derived from a polynucleotide fragment from *Zymomonas mobilis*, and more preferably, is the sequence provided in SEQ ID NO: 1, or a fragment of that sequence.

In even another embodiment, the host cell of the above aspect and foregoing embodiments is ethanologenic.

In a second aspect, the present invention provides a recombinant ethanologenic host cell containing a heterologous polynucleotide segment that encodes a polysaccharase and this segment is under the transcriptional control of an exogenous surrogate promoter.

In one embodiment, the host cell is a bacterial cell, preferably Gram-negative, facultatively anaerobic, and from the family Enterobacteriaceae. In a related embodiment, the recombinant ethanologenic host cell is of the genus *Escherichia* or *Klebsiella* and, preferably, is the strain *E. coli* B, *E. coli* DH5α, *E. coli* KO4 (ATCC 55123), *E. coli* KO11 (ATCC 55124), *E. coli* KO12 (ATCC 55125), *E. coli* LY01, *K. oxytoca* M5A1, or *K. oxytoca* P2 (ATCC 55307).

In another embodiment, the recombinant host cell contains a polynucleotide segment that encodes a polysaccharase that is a glucanase, endoglucanase, exoglucanase, cellobiohydrolase, α-glucosidase, endo-1,4-α-xylanase, β-xylosidase, β-glucuronidase, α-L-arabinofuranosidase, acetylesterase, acetylxylanesterase, α-amylase, β-amylase, glucoamylase, pullulanase, β-glucanase, hemicellulase, arabinosidase, mannanase, pectin hydrolase, pectate lyase, or a combination of these polysaccharases. In a related embodiment, the polysaccharase is a glucanase, preferably an expression product of a *celZ* gene, and more preferably, derived from *Erwinia chrysanthemi*.

In another embodiment, the surrogate promoter for driving gene expression in the recombinant host cell is derived from a polynucleotide fragment from *Zymomonas mobilis*, and more preferably, is the sequence provided in SEQ ID NO: 1, or is a fragment of that sequence.

In another preferred embodiment, the above aspect and foregoing embodiments features a host cell that is ethanologenic.

In a third aspect, the invention features a recombinant ethanologenic Gram-negative bacterial host cell containing a first heterologous polynucleotide segment containing a sequence encoding a first polypeptide and a second heterologous polynucleotide segment containing a sequence encoding a secretory polypeptide/s where the first heterologous polysaccharide is under the transcriptional control of a surrogate promoter and the production of the first polypeptide by the host cell is increased.

In one embodiment, the first polypeptide is secreted.

In another embodiment, the recombinant host cell is a facultatively anaerobic bacterial cell. In a related embodiment, the host cell is from the family Enterobacteriaceae, preferably *Escherichia* or *Klebsiella*, and more preferably, is the strain *E. coli* B, *E. coli* DH5α, *E. coli* KO4 (ATCC 55123), *E. coli* KO11 (ATCC 55124), *E. coli* KO12 (ATCC 55125), or *E. coli* LY01, *K. oxytoca* M5A1, or *K. oxytoca* P2 (ATCC 55307).

In another embodiment, the first polypeptide of the recombinant host is a polysaccharase, and, preferably the polypeptide is of increased activity. In a related embodiment, the polysaccharase is a glucanase, endoglucanase, exoglucanase, cellobiohydrolase, α-glucosidase, endo-1,4-α-xylanase, β-xylosidase, β-glucuronidase, α-L-arabinofuranosidase, acetylesterase, acetylxylanesterase, α-amylase, β-amylase,

glucoamylase, pullulanase, β-glucanase, hemicellulase, arabinosidase, mannanase, pectin hydrolase, pectate lyase, or a combination of these polysaccharases.

In a preferred embodiment, the first polypeptide of the recombinant host is the polysaccharase glucanase, preferably an expression product of the *celZ* gene, and more preferably, is derived from *Erwinia chrysanthemi*.

In another embodiment, the second heterologous polynucleotide segment of the recombinant host cell contains at least one *pul* gene or *out* gene, preferably derived from a bacterial cell from the family Enterobacteriaceae and more preferably, from *K. oxytoca*, *E. carotovora*, *E. carotovora* subspecies *carotovora*, *E. carotovora* subspecies *atroseptica*, or *E. chrysanthemi*.

In a fourth aspect, the invention provides a method for enzymatically degrading an oligosaccharide. The method involves contacting an oligosaccharide with a host cell containing a first heterologous polynucleotide segment containing a sequence encoding a polysaccharase that is under the transcriptional control of a surrogate promoter. Moreover, the surrogate promoter is capable of causing increased production of the polysaccharase. In addition, the recombinant host cell of the above method also contains a second heterologous polynucleotide segment containing a sequence encoding a secretory polypeptide. The expression of the first and second polynucleotide segments of the host cell of this aspect result in the production of an increased amount of polysaccharase activity such that the oligosaccharide is enzymatically degraded. In a preferred embodiment, the polysaccharase is secreted.

In one embodiment of the above aspect, the host cell is ethanologenic. In another embodiment, the method is carried out in an aqueous solution. In even another embodiment, the method is used for simultaneous saccharification and fermentation. In still another embodiment, the oligosaccharide is preferably lignocellulose, hemicellulose, cellulose, pectin, or any combination of these oligosaccharides.

In a fifth aspect, the invention features a method of identifying a surrogate promoter capable of increasing the expression of a gene-of-interest in a host cell. The method involves fragmenting a genomic polynucleotide from an organism into one or more fragments and placing a gene-of-interest under the transcriptional control of at least one of these fragments. The method further involves introducing such a fragment and gene-of-interest into a host cell and identifying a host cell having increased

production of the gene-of-interest such that the increased expression indicates that the fragment is a surrogate promoter.

In a sixth aspect, the invention provides a method of making a recombinant host cell for use in simultaneous saccharification and fermentation. In particular, the method involves introducing into the host cell a first heterologous polynucleotide segment containing a sequence encoding a polysaccharase polypeptide under the transcriptional control of a surrogate promoter, the promoter being capable of causing increased expression of the polysaccharase polypeptide. In addition, the method further includes introducing into the host cell a second heterologous polynucleotide segment containing a sequence encoding a secretory polypeptide/s such that the expression of the first and second polynucleotide segments results in the increased production of a polysaccharase polypeptide by the recombinant host cell. In a preferred embodiment, the increased production of the polysaccharase polypeptide is an increase in activity, amount, or a combination thereof. In another preferred embodiment, the polysaccharase polypeptide is secreted. In a more preferred embodiment, the host cell is ethanologenic.

In a seventh aspect, the invention features a vector comprising the sequence of pLOI2306 (SEQ ID NO: 12).

In an eighth aspect, the invention features a host cell comprising the foregoing vector.

In a ninth aspect, the invention features a method of making a recombinant host cell integrant including the steps of introducing into the host a vector comprising the sequence of pLOI2306 and identifying a host cell having the vector stably integrated.

In a tenth aspect, the invention features a method for expressing a polysaccharase in a host cell encompassing the steps of introducing into the host cell a vector containing the polynucleotide sequence of pLOI2306 and identifying a host cell expressing the polysaccharase. In a preferred embodiment, each of the above aspects features a host cell that is ethanologenic.

In an eleventh aspect, the invention provides a method for producing ethanol from an oligosaccharide source by contacting said oligosaccharide source with a ethanologenic host cell containing a first heterologous polynucleotide segment comprising a sequence encoding a polysaccharase under the transcriptional control of a surrogate promoter. Moreover, the promoter is capable of causing increased expression

of the polysaccharase. In addition, the ethanologenic host contains a second heterologous polynucleotide segment comprising a sequence encoding a secretory polypeptide. The expression of said first and second polynucleotide segments of the ethanologenic host cell result in the increased production of polysaccharase activity by the host cell such that the oligosaccharide source is enzymatically degraded and fermented into ethanol.

In one embodiment, the first polypeptide of the recombinant host is a polysaccharase, and, preferably the polypeptide is of increased activity. In a related embodiment, the polysaccharase is a glucanase, endoglucanase, exoglucanase, cellobiohydrolase, α-glucosidase, endo-1,4-α-xylanase, β-xylosidase, β-glucuronidase, α-L-arabinofuranosidase, acetylesterase, acetylxylanesterase, α-amylase, β-amylase, glucoamylase, pullulanase, β-glucanase, hemicellulase, arabinosidase, mannanase, pectin hydrolase, pectate lyase, or a combination of these polysaccharases.

In a preferred embodiment, the first polypeptide of the recombinant host is the polysaccharase glucanase, preferably an expression product of the *celZ* gene, and more preferably, is derived from *Erwinia chrysanthemi*.

In another embodiment, the second heterologous polynucleotide segment of the recombinant host cell contains at least one *pul* gene or *out* gene, preferably derived from a bacterial cell from the family Enterobacteriaceae and more preferably, from *K. oxytoca*, *E. carotovora*, *E. carotovora* subspecies *carotovora*, *E. carotovora* subspecies *atroseptica*, or *E. chrysanthemi*.

In another embodiment, the recombinant host cell is a facultatively anaerobic bacterial cell. In a related embodiment, the host cell is from the family Enterobacteriaceae, preferably *Escherichia* or *Klebsiella*, and more preferably, is the strain *E. coli* KO4 (ATCC 55123), *E. coli* KO11 (ATCC 55124), *E. coli* KO12 (ATCC 55125), *K. oxytoca* M5A1, or *K. oxytoca* P2 (ATCC 55307).

In another embodiment, the method is carried out in an aqueous solution. In even another embodiment, the method is used for simultaneous saccharification and fermentation. In still another embodiment, the oligosaccharide is preferably lignocellulose, hemicellulose, cellulose, pectin, or any combination of these oligosaccharides.

In yet another embodiment, the method uses a nucleic acid construct that is, or is derived from, a plasmid selected from the group consisting of pLOI2306.

Other features and advantages of the invention will be apparent from the following detailed description and claims.

Brief Description of the Drawings

Figure 1 shows fermentation rates for the ethanologenic recombinant host *E. coli* KO11 using rice hull substrates pretreated with dilute acid and supplemented with two different medias.

Figure 2 shows simultaneous saccharification and fermentation (SSF) rates for the ethanologenic recombinant host strain *K. oxytoca* P2 using mixed waste office paper. Insoluble residues from SSF were recycled as a source of bound cellulase enzymes and substrate during subsequent fermentations.

Figure 3 shows the structure of the plasmid pLOI2171, a low copy promoter probe vector showing the orientation of the kanamycin resistance gene (*kan*) for selection, the temperature sensitive pSC101 replicon (Rep(ts)) for episomal maintenance of the plasmid, and the promoterless polysaccharase gene *celZ* encoding glucanase (EGZ).

Figure 4 is a graph showing the high correspondence between the size of the zone of clearance on CMC indicator plates (x-axis) measured for a transformed bacterial colony and the amount of glucanase activity expressed (y-axis).

Figure 5 shows the partial nucleotide sequence (SEQ ID NO: 1) of the *Z. mobilis* DNA fragment in the pLOI2183 plasmid that functions as a surrogate promoter. The full sequence has been assigned GenBank accession number AF109242 (SEQ ID NO: 2). Indicated are two transcriptional start sites (#) , -35 and -10 regions, the Shine-Delgarno site (bold), partial vector and *celZ* sequence (lowercase), and the *celZ* start codon (atg indicated in bold).

Figure 6 represents electron micrographs of *E. coli* B cells harboring different plasmids expressing little if any (pUC19; panel A), moderate (pLOI2164; panel B), and high levels (pLOI2307; panel C) of glucanase in the form of periplasmic inclusion bodies (pib) localized between the outer cell wall and the inner membrane (im). The bar shown represents 0.1 μm.

Figure 7 shows a schematic detailing the cloning strategy used to construct the *celZ* integration vector pLOI2306, a genetic construct capable of being introduced into the genome of a recombinant host and conferring stable glucanase expression activity to the host.

Figure 8 shows a schematic representation of the *celZ* integration vector pLOI2306 (SEQ ID NO: 12) with the locations of the surrogate promoter from *Z. mobilis*, the *celZ* gene from *E. chrysanthemi*, resistance markers (*bla* and *tet*), and *K. oxytoca* target sequence indicated.

Detailed Description of the Invention

In order for the full scope of the invention to be clearly understood, the following definitions are provided.

I. Definitions

As used herein the term "recombinant host" is intended to include a cell suitable for genetic manipulation, *e.g.*, which can incorporate heterologous polynucleotide sequences, *e.g.*, which can be transfected. The cell can be a microorganism or a higher eukaryotic cell. The term is intended to include progeny of the cell originally transfected. In preferred embodiments, the cell is a bacterial cell, *e.g.*, a Gram-negative bacterial cell, and this term is intended to include all facultatively anaerobic Gram-negative cells of the family Enterobacteriaceae such as *Escherichia, Shigella, Citrobacter, Salmonella, Klebsiella, Enterobacter, Erwinia, Kluyvera, Serratia, Cedecea, Morganella, Hafnia, Edwardsiella, Providencia, Proteus,* and *Yersinia.* Particularly preferred recombinant hosts are *Escherichia coli* or *Klebsiella oxytoca* cells.

The term "heterologous polynucleotide segment" is intended to include a polynucleotide segment that encodes one or more polypeptides or portions or fragments of polypeptides. A heterologous polynucleotide segment may be derived from any source, *e.g.*, eukaryotes, prokaryotes, virii, or synthetic polynucleotide fragments.

The terms "polysaccharase" or "cellulase" are used interchangeably herein and are intended to include a polypeptide capable of catalyzing the degradation or depolymerization of any linked sugar moiety, *e.g.*, disaccharides, trisaccharides, oligosaccharides, including, complex carbohydrates, *e.g.*, lignocellulose, which

comprises cellulose, hemicellulose, and pectin. The terms are intended to include cellulases such as glucanases, including both endoglucanases and exoglucanases, and β-glucosidase. More particularly, the terms are intended to include, *e.g.*, cellobiohydrolase, endo-1,4-β-xylanase, β-xylosidase, α-glucuronidase, α-L-arabinofuranosidase, acetylesterase, acetylxylanesterase, α-amylase, β-amylase, glucoamylase, pullulanase, β-glucanase, hemicellulase, arabinosidase, mannanase, pectin hydrolase, pectate lyase, or a combination of any of these cellulases.

The term "surrogate promoter" is intended to include a polynucleotide segment that can transcriptionally control a gene-of-interest that it does not transcriptionally control in nature. In a preferred embodiment, the transcriptional control of a surrogate promoter results in an increase in expression of the gene-of-interest. In a preferred embodiment, a surrogate promoter is placed 5' to the gene-of-interest. A surrogate promoter may be used to replace the natural promoter, or may be used in addition to the natural promoter. A surrogate promoter may be endogenous with regard to the host cell in which it is used or it may be a heterologous polynucleotide sequence introduced into the host cell, *e.g.*, exogenous with regard to the host cell in which it is used.

The terms "oligosaccharide source," "oligosaccharide," "complex cellulose," "complex carbohydrate," and "polysaccharide" are used essentially interchangeably and are intended to include any carbohydrate source comprising more than one sugar molecule. These carbohydrates may be derived from any unprocessed plant material or any processed plant material. Examples are wood, paper, pulp, plant derived fiber, or synthetic fiber comprising more than one linked carbohydrate moiety, *i.e.*, one sugar residue. One particular oligosaccharide source is lignocellulose which represents approximately 90% of the dry weight of most plant material and contains carbohydrates, *e.g.*, cellulose, hemicellulose, pectin, and aromatic polymers, *e.g.*, lignin. Cellulose, makes up 30%-50% of the dry weight of lignocellulose and is a homopolymer of cellobiose (a dimer of glucose). Similarly, hemicellulose, makes up 20%-50% of the dry weight of lignocellulose and is a complex polymer containing a mixture of pentose (xylose, arabinose) and hexose (glucose, mannose, galactose) sugars which contain acetyl and glucuronyl side chains. Pectin makes up 1%-20% of the dry weight of lignocellulose and is a methylated homopolymer of glucuronic acid. Any one or a combination of the above carbohydrate polymers are potential sources of sugars for

depolymerization and subsequent bioconversion to ethanol by fermentation according to the products and methods of the present invention.

The term "gene/s" is intended to include nucleic acid molecules, *e.g.*, polynucleotides which include an open reading frame encoding a polypeptide, and can further include non-coding regulatory sequences, and introns. In addition, the term gene/s is intended to include one or more genes that map to a functional locus, *e.g.*, the *out* or *pul* genes of *Erwinia* and *Klebsiella*, respectively, that encode more than one gene product, *e.g.*, a secretory polypeptide.

The term "gene-of-interest" is intended to include a specific gene for a selected purpose. The gene may be endogenous to the host cell or may be recombinantly introduced into the host cell. In a preferred embodiment, a gene-of-interest is involved in at least one step in the bioconversion of a carbohydrate to ethanol. Accordingly, the term is intended to include any gene encoding a polypeptide such as an alcohol dehydrogenase, a pyruvate decarboxylase, a secretory protein/s, or a polysaccharase, *e.g.*, a glucanase, such as an endoglucanase or exoglucanase, a cellobiohydrolase, β-glucosidase, endo-1,4-β-xylanase, β-xylosidase, α-glucuronidase, α-L-arabinofuranosidase, acetylesterase, acetylxylanesterase, α-amylase, β-amylase, glucoamylase, pullulanase, β-glucanase, hemicellulase, arabinosidase, mannanase, pectin hydrolase, pectate lyase, or a combination thereof.

The term "fragmenting a genomic polynucleotide from an organism" is intended to include the disruption of the genomic polynucleotide belonging to an organism into one or more segments using either mechanical, *e.g.*, shearing, sonication, trituration, or enzymatic methods, *e.g.*, a nuclease. Preferably, a restriction enzyme is used in order to facilitate the cloning of genomic fragments into a test vector for subsequent identification as a candidate promoter element. A genomic polynucleotide may be derived from any source, *e.g.*, eukaryotes, prokaryotes, virii, or synthetic polynucleotide fragments.

The term "simultaneous saccharification and fermentation" or "SSF" is intended to include the use of one or more recombinant hosts for the contemporaneous degradation or depolymerization of a complex sugar and bioconversion of that sugar residue into ethanol by fermentation.

The term "transcriptional control" is intended to include the ability to modulate gene expression at the level of transcription. In a preferred embodiment, transcription. and thus gene expression, is modulated by replacing or adding a surrogate promoter near the 5' end of the coding region of a gene-of-interest thereby resulting in altered gene expression.

The term "expression" is intended to include the expression of a gene at least at the level of RNA production.

The term "expression product" is intended to include the resultant product of an expressed gene, *e.g.*, a polypeptide.

The term "increased expression" is intended to include an alteration in gene expression at least at the level of increased RNA production and preferably, at the level of polypeptide expression.

The term "increased production" is intended to include an increase in the amount of a polypeptide expressed, in the level of the enzymatic activity of the polypeptide, or a combination thereof.

The terms "activity" and "enzymatic activity" are used interchangeably and are intended to include any functional activity normally attributed to a selected polypeptide when produced under favorable conditions. The activity of a polysaccharase would be, for example, the ability of the polypeptide to enzymatically depolymerize a complex saccharide. Typically. the activity of a selected polypeptide encompasses the total enzymatic activity associated with the produced polypeptide. The polypeptide produced by a host cell and having enzymatic activity may be located in the intracellular space of the cell, cell-associated, secreted into the extracellular milieu. or a combination thereof. Techniques for determining total activity as compared to secreted activity are described herein and are known in the art.

The term "secreted" is intended to include an increase in the secretion of a polypeptide, *e.g.*, a heterologous polypeptide, preferably a polysaccharase. Typically, the polypeptide is secreted at an increased level that is in excess of the naturally-occurring amount of secretion. More preferably, the term "secreted" refers to an increase in secretion of a given polypeptide that is at least 10% and more preferably, at least 100%, 200%, 300,%, 400%, 500%, 600%, 700%, 800%, 900%, 1000%, or more, as compared to the naturally-occurring level of secretion.

The term "secretory polypeptide" is intended to include any polypeptide/s, alone or in combination with other polypeptides, that facilitate the transport of another polypeptide from the intracellular space of a cell to the extracellular milieu. In one embodiment, the secretory polypeptide/s encompass all the necessary secretory polypeptides sufficient to impart secretory activity to a Gram-negative host cell. Typically, secretory proteins are encoded in a single region or locus that may be isolated from one host cell and transferred to another host cell using genetic engineering. In a preferred embodiment, the secretory polypeptide/s are derived from any bacterial cell having secretory activity. In a more preferred embodiment, the secretory polypeptide/s are derived from a host cell having Type II secretory activity. In another more preferred embodiment, the host cell is selected from the family Enterobacteriaceae. In a most preferred embodiment, the secretory polypeptide/s are one or more gene products of the *out* or *pul* genes derived from, respectively, *Erwinia* or *Klebsiella.* Moreover, the skilled artisan will appreciate that any secretory protein/s derived from a related host that is sufficiently homologous to the *out* or *pul* gene/s described herein may also be employed (Pugsley *et al.*, (1993) *Microbiological Reviews* 57:50-108; Lindeberg *et al.*, (1996) *Mol. Micro.* 20:175-190; Lindeberg *et al.*, (1992) *J. of Bacteriology* 174:7385-7397; He *et al.*, (1991) *Proc. Natl. Acad. Sci. USA*, 88:1079-1083).

The term "derived from" is intended to include the isolation (in whole or in part) of a polynucleotide segment from an indicated source. The term is intended to include, for example, direct cloning, PCR amplification, or artificial synthesis from, or based on, a sequence associated with the indicated polynucleotide source.

The term "ethanologenic" is intended to include the ability of a microorganism to produce ethanol from a carbohydrate as a primary fermentation product. The term is intended to include naturally occurring ethanologenic organisms, ethanologenic organisms with naturally occurring or induced mutations, and ethanologenic organisms which have been genetically modified.

The term "Gram-negative bacteria" is intended to include the art recognized definition of this term. Typically, Gram-negative bacteria include, for example, the family Enterobacteriaceae which comprises, among others, the species *Escherichia* and *Klebsiella.*

The term "sufficiently homologous" is intended to include a first amino acid or nucleotide sequence which contains a sufficient or minimum number of identical or equivalent amino acid residues or nucleotides, *e.g.*, an amino acid residue which has a similar side chain, to a second amino acid or nucleotide sequence such that the first and second amino acid or nucleotide sequences share common structural domains and/or a common functional activity. For example, amino acid or nucleotide sequences which share common structural domains have at least about 40% homology, preferably 50% homology, more preferably 60%, 70%, 80%, or 90% homology across the amino acid sequences of the domains and contain at least one, preferably two, more preferably three, and even more preferably four, five, or six structural domains, are defined herein as sufficiently homologous. Furthermore, amino acid or nucleotide sequences which share at least 40%, preferably 50%, more preferably 60%, 70%, 80%, or 90% homology and share a common functional activity are defined herein as sufficiently homologous.

In one embodiment, two polynucleotide segments, *e.g.*, promoters, are "sufficiently homologous" if they have substantially the same regulatory effect as a result of a substantial identity in nucleotide sequence. Typically, "sufficiently homologous" sequences are at least 50%, more preferably at least 60%, 70%, 80%, or 90% identical, at least in regions known to be involved in the desired regulation. More preferably, no more than five bases differ. Most preferably, no more than five consecutive bases differ.

To determine the percent identity of two polynucleotide segments, or two amino acid sequences, the sequences are aligned for optimal comparison purposes (*e.g.*, gaps can be introduced in one or both of a first and a second amino acid or nucleic acid sequence for optimal alignment and non-homologous sequences can be disregarded for comparison purposes). In a preferred embodiment, the length of a reference sequence aligned for comparison purposes is at least 30%, preferably at least 40%, more preferably at least 50%, even more preferably at least 60%, and even more preferably at least 70%, 80%, or 90% of the length of the reference sequence. The amino acid residues or nucleotides at corresponding amino acid positions or nucleotide positions are then compared. When a position in the first sequence is occupied by the same amino acid residue or nucleotide as the corresponding position in the second sequence, then the molecules are identical at that position (as used herein amino acid or nucleic acid

"identity" is equivalent to amino acid or nucleic acid "homology"). The percent identity between the two sequences is a function of the number of identical positions shared by the sequences, taking into account the number of gaps, and the length of each gap, which need to be introduced for optimal alignment of the two sequences.

The comparison of sequences and determination of percent identity between two sequences can be accomplished using a mathematical algorithm. In a preferred embodiment, the percent identity between two amino acid sequences is determined using the Needleman and Wunsch (*J. Mol. Biol.* (48):444-453 (1970)) algorithm which has been incorporated into the GAP program in the GCG software package (available at http://www.gcg.com), using either a Blossom 62 matrix or a PAM250 matrix, and a gap weight of 16, 14, 12, 10, 8, 6, or 4 and a length weight of 1, 2, 3, 4, 5, or 6. In yet another preferred embodiment, the percent identity between two nucleotide sequences is determined using the GAP program in the GCG software package (available at http://www.gcg.com), using a NWSgapdna.CMP matrix and a gap weight of 40, 50, 60, 70, or 80 and a length weight of 1, 2, 3, 4, 5, or 6. In another embodiment, the percent identity between two amino acid or nucleotide sequences is determined using the algorithm of E. Meyers and W. Miller (CABIOS, 4:11-17 (1989)) which has been incorporated into the ALIGN program (version 2.0), using a PAM120 weight residue table, a gap length penalty of 12 and a gap penalty of 4.

The polynucleotide and amino acid sequences of the present invention can further be used as a "query sequence" to perform a search against public databases to, for example, identify other family members or related sequences, *e.g.*, promoter sequences. Such searches can be performed using the NBLAST and XBLAST programs (version 2.0) of Altschul, *et al.* (1990) *J. Mol. Biol.* 215:403-10. BLAST nucleotide searches can be performed with the NBLAST program, score = 100, wordlength = 12 to obtain nucleotide sequences homologous to polynucleotide molecules of the invention. BLAST protein searches can be performed with the XBLAST program, score = 50, wordlength = 3 to obtain amino acid sequences homologous to polypeptide molecules of the invention. To obtain gapped alignments for comparison purposes, Gapped BLAST can be utilized as described in Altschul *et al.*, (1997) *Nucleic Acids Res.* 25(17):3389-3402. When utilizing BLAST and Gapped BLAST programs, the default parameters of

the respective programs (*e.g.*, XBLAST and NBLAST) can be used. See http://www.ncbi.nlm.nih.gov.

II. Recombinant Hosts

The present invention relates to recombinant host cells that are suitable for use in the production of ethanol. In one embodiment, the cell comprises a heterologous, polynucleotide segment encoding a polypeptide under the transcriptional control of a surrogate promoter. The heterologous polynucleotide and surrogate promoter may be plasmid-based or integrated into the genome of the organism (as described in the examples). In a preferred embodiment, the host cell is used as a source of a desired polypeptide for use in the bioconversion of a complex sugar to ethanol, or a step thereof.

In a preferred embodiment, the heterologous polynucleotide segment encodes a polysaccharase polypeptide which is expressed at higher levels than are naturally occurring in the host. The polysaccharase may be a β-glucosidase, a glucanase, either an endoglucanase or a exoglucanase, cellobiohydrolase, endo-1,4-β-xylanase, β-xylosidase, α-glucuronidase, α-L-arabinofuranosidase, acetylesterase, acetylxylanesterase, α-amylase, β-amylase, glucoamylase, pullulanase, β-glucanase, hemicellulase, arabinosidase, mannanase, pectin hydrolase, pectate lyase, or a combination thereof.

In one embodiment, the polysaccharase is derived from *E. chrysanthemi* and is the glucanase (EGZ) polypeptide encoded by the *celZ* gene. However, other polysaccharases from *E. chrysanthemi* may be used including, *e.g.*, the glucohydrolases encoded by *celY* (Guiseppi *et al.*, (1991) *Gene* 106:109-114) or *bgxA* (Vroeman *et al.*, (1995) *Mol. Gen. Genet.* 246:465-477). The *celY* gene product (EGY) is an endoglucanase. The *bgxA* gene encodes β-glucosidase and β-xylosidase activities (Vroeman *et al.*, (1995) *Mol. Gen. Genet.* 246:465-477). Preferably, an increase in polysaccharase activity of at least 10%, more preferably 20%, 30%, 40%, or 50% is observed. Most preferably, an increase in polysaccharase activity of several fold is obtained, *e.g.*, 200%, 300%, 400%, 500%, 600%, 700%, or 800%.

Alternatively, a desired polysaccharase may be encoded by a polynucleotide segment from another species, *e.g.*, a yeast, an insect, an animal, or a plant. Any one or more of these genes may be introduced and expressed in the host cell of the invention in

order to give rise to elevated levels of a polysaccharase suitable for depolymerizing a complex sugar substrate. The techniques for introducing and expressing one of these genes in a recombinant host, are presented in the examples.

In another embodiment of the invention, the host cell has been engineered to express a secretory protein/s to facilitate the export of a desired polypeptide from the cell. In one embodiment, the secretory protein or proteins are derived from a Gram-negative bacterial cell, *e.g.*, a cell from the family Enterobacteriaceae. In another embodiment, the secretory protein/s are from *Erwinia* and are encoded by the *out* genes. In another embodiment, the secretory proteins are the *pul* genes derived from *Klebsiella*. The introduction of one or more of these secretory proteins is especially desirable if the host cell is an enteric bacterium, *e.g.*, a Gram-negative bacterium having a cell wall. Representative Gram-negative host cells of the invention are from the family Enterobacteriaceae and include, *e.g.*, *Escherichia* and *Klebsiella*. In one embodiment, the introduction of one or more secretory proteins into the host results in an increase in the secretion of the selected protein, *e.g.*, a polysaccharase, as compared to naturally-occurring levels of secretion. Preferably, the increase in secretion is at least 10% and more preferably, 100%, 200%, 300%, 400%, 500%, 600%, 700%, 800%, 900%, 1000%, or more, as compared to naturally-occurring levels of secretion. In a preferred embodiment, the addition of secretion genes allows for the polysaccharase polypeptide to be produced at higher levels. In a preferred embodiment, the addition of secretion genes allows for the polysaccharase polypeptide to be produced with higher enzymatic activity. In a most preferred embodiment, the polysaccharase is produced at higher levels and with higher enzymatic activity. Preferably, an increase in polysaccharase activity of at least 10%, more preferably 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90%, or 100% is observed. Most preferably, an increase in polysaccharase activity of several fold is obtained, *e.g.*, 200%, 300%, 400%, 500%, 600%, 700%, 800%, 900%, or 1000%, as compared to cells without secretion genes (*e.g.*, cells that either lack or do not express secretion genes at a sufficient level). The techniques and methods for introducing such genes and measuring increased output of a desired polypeptide such as, *e.g.*, a polysaccharase, are described in further detail in the examples. Other equivalent methods are known to those skilled in the art.

In preferred embodiments, the invention makes use of a recombinant host that is also ethanologenic. In one embodiment, the recombinant host is a Gram-negative bacterium. In another embodiment, the recombinant host is from the family Enterobacteriaceae. The ethanologenic hosts of U.S.P.N. 5,821,093, hereby incorporated by reference, for example, are suitable hosts and include, in particular, *E. coli* strains KO4 (ATCC 55123), KO11 (ATCC 55124), and KO12 (ATCC 55125), and *Klebsiella oxytoca* strain M5A1. Alternatively, a non-ethanologenic host of the present invention may be converted into an ethanologenic host (such as the above-mentioned strains) by introducing, for example, ethanologenic genes from an efficient ethanol producer like *Zymomonas mobilis*. This type of genetic engineering, using standard techniques, results in a recombinant host capable of efficiently fermenting sugar into ethanol. In addition, the LY01 ethanol tolerant strain (ATCC _____) may be employed as described in published PCT international application WO 98/45425 and this published application is hereby incorporated by reference (see also, *e.g.*, Yomano *et al.* (1998) *J. of Ind. Micro. & Bio.* 20:132-138).

In another preferred embodiment, the invention makes use of a non-ethanologenic recombinant host, *e.g.*, *E. coli* strain B, *E. coli* strain DH5α, or *Klebsiella oxytoca* strain M5A1. These strains may be used to express a desired polypeptide, *e.g.*, a polysaccharase using techniques describe herein. In addition, these recombinant host may be used in conjunction with another recombinant host that expresses, yet another desirable polypeptide, *e.g.*, a different polysaccharase. In addition, the non-ethanologenic host cell may be used in conjunction with an ethanologenic host cell. For example, the use of a non-ethanologenic host/s for carrying out, *e.g.*, the depolymerization of a complex sugar may be followed by the use of an ethanologenic host for fermenting the depolymerized sugar. Accordingly, it will be appreciated that these reactions may be carried out serially or contemporaneously using, *e.g.*, homogeneous or mixed cultures of non-ethanologenic and ethanologenic recombinant hosts.

In a preferred embodiment, one or more genes necessary for fermenting a sugar substrate into ethanol are provided on a plasmid or integrated into the host chromosome. More preferably, essential genes for fermenting a sugar substrate into ethanol, *e.g.*, pyruvate decarboxylase (*e.g.*, *pdc*) and/or alcohol dehydrogenase (*e.g.*, *adh*) are

introduced into the host of the invention using an artificial operon such as the PET operon as described in U.S.P.N. 5,821,093, hereby incorporated by reference. Indeed, it will be appreciated that the present invention, in combination with what is known in the art, provides techniques and vectors for introducing multiple genes into a suitable host (see, *e.g.*, *Current Protocols in Molecular Biology*, eds. Ausubel *et al.*, John Wiley & Sons (1992), Sambrook, J. *et al.*, *Molecular Cloning: A Laboratory Manual. 2nd, ed.*, *Cold Spring Harbor Laboratory,* Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY (1989), and *Bergey's Manual of Determinative Bacteriology*, Kreig *et al.*, Williams and Wilkins (1984), hereby incorporated by reference). Accordingly, using the methods of the invention, a single genetic construct could encode all of the necessary gene products (*e.g.*, a glucanase, an endoglucanase, an exoglucanase, a secretory protein/s, pyruvate decarboxylase, alcohol dehydrogenase) for performing simultaneous saccharification and fermentation (SSF). In addition, it will also be appreciated that such a host may be further manipulated, using methods known in the art, to have mutations in any endogenous gene/s (*e.g.*, recombinase genes) that would interfere with the stability, expression, and function of the introduced genes. Further, it will also be appreciated that the invention is intended to encompass any regulatory elements, gene/s, or gene products, *i.e.*, polypeptides, that are sufficiently homologous to the ones described herein.

Methods for screening strains having the introduced genes are routine and may be facilitated by visual screens that can identify cells expressing either the alcohol dehydrogenase (ADH) or glucanase (EGZ) gene product. The ADH gene product produces acetaldehyde that reacts with the leucosulfonic acid derivative of p-roseaniline to produce an intensely red product. Thus, ADH-positive clones can be easily screened and identified as bleeding red colonies. Methods for screening for EGZ, *e.g.*, polysaccharase activity, also results in a clear visual phenotype as described below and in the examples.

Recombinant bacteria expressing, for example, the PET operon typically grow to higher cell densities in liquid culture than the unmodified parent organisms due to the production of neutral rather than acidic fermentation products (Ingram *et al.*, (1988) *Appl. Environ. Microbiol.* 54:397-404). On plates, ethanologenic clones are readily apparent as large, raised colonies which appear much like yeast. These traits have been

very useful during the construction of new strains and can provide a preliminary indication of the utility of new constructs. Rapid evaluations of ethanol producing potential can also be made by testing the speed of red spot development on aldehyde indicator plates (Conway *et al.*, (1987) *J. Bacteriol.* 169:2591-2597). Typically, strains which prove to be efficient in sugar conversion to ethanol can be recognized by the production of red spots on aldehyde indicator plates within minutes of transfer.

In a most preferred embodiment of the invention, a single host cell is ethanologenic, that is, has all the necessary genes, either naturally occurring or artificially introduced or enhanced (*e.g.*, using a surrogate promoter and/or genes from a different species or strain), such that the host cell has the ability to produce and secrete a polysaccharase/s, degrade a complex sugar, and ferment the degraded sugar into ethanol. Accordingly, such a host is suitable for simultaneous saccharification and fermentation.

Moreover, the present invention takes into account that the native *E. coli* fermentation pathways produce a mixture of acidic and neutral products (in order of abundance): lactic acid, hydrogen + carbon dioxide (from formate), acetic acid, ethanol, and succinate. However, the *Z. mobilis* PDC (pyruvate decarboxylase) has a lower Km for pyruvate than any of the competing *E. coli* enzymes. By expressing high activities of PDC, carbon flow is effectively redirected from lactic acid and acetyl-CoA into acetylaldehyde and ethanol. Small amounts of phosphoenolpyruvate can be eliminated by deleting the fumarate reductase gene (*frd*) (Ingram *et al.*, (1991) U.S.P.N, 5,000,000; Ohta *et al.*, (1991) *Appl. Environ. Microbiol.* 57:893-900). Additional mutations (*e.g.*, in the *pfl* or *ldh* genes) may be made to completely eliminate other competing pathways (Ingram *et al.*, (1991) U.S.P.N, 5,000,000). Additional mutations to remove enzymes (*e.g.*, recombinases, such as *recA*) that may compromise the stability of the introduced genes (either plasmid-based or integrated into the genome) may also be introduced, selected for, or chosen from a particular background.

In addition, it should be readily apparent to one skilled in the art that the ability conferred by the present invention, to transform genes coding for a protein or an entire metabolic pathway into a single manipulable construct, is extremely useful. Envisioned in this regard, for example, is the application of the present invention to a variety of situations where genes from different genetic loci are placed on a chromosome. This

may be a multi-cistronic cassette under the control of a single promoter or separate promoters may be used.

Exemplary *E. coli* strains that are ethanologenic and suitable for further improvement according to the methods of the invention include, for example, KO4, KO11, and KO12 strains, as well as the LY01 strain, an ethanol-tolerant mutant of the *E. coli* strain KO11. Ideally, these strains may be derived from the *E. coli* strain ATCC 11303, which is hardy to environmental stresses and can be engineered to be ethanologenic and secrete a polysaccharase/s. In addition, recent PCR investigations have confirmed that the ATCC 11303 strain lacks all genes known to be associated with the pathogenicity of *E. coli* (Kuhnert *et al.*, (1997) *Appl. Environ. Microbiol.* 63:703-709).

Another preferred ethanologenic host for improvement according to the methods of the invention is the *E. coli* KO11 strain which is capable of fermenting hemicellulose hydrolysates from many different lignocellulosic materials and other substrates (Asghari *et al.*, (1996) *J. Ind. Microbiol.* 16:42-47; Barbosa *et al.*, (1992) *Current Microbiol.* 28:279-282; Beall *et al.*, (1991) *Biotechnol. Bioeng.* 38:296-303; Beall *et al.*, (1992) *Biotechnol. Lett.* 14:857-862; Hahn-Hagerdal *et al.*, (1994) *Appl. Microbiol. Biotechnol.* 41:62-72; Moniruzzaman *et al.*, (1996) *Biotechnol. Lett.* 18:955-990; Moniruzzaman *et al.*, (1998) *Biotechnol. Lett.* 20:943-947; Grohmann *et al.*, (1994) *Biotechnol. Lett.* 16:281-286; Guimaraes *et al.*, (1992) *Biotechnol. Bioeng.* 40:41-45; Guimaraes *et al.*, (1992) *Biotechnol. Lett.* 14:415-420; Moniruzzaman *et al.*, (1997) *J. Bacteriol.* 179:1880-1886). In Figure 1, the kinetics of bioconversion for this strain are shown. In particular, this strain is able to rapidly ferment a hemicellulose hydrolysate from rice hulls (which contained 58.5 g/L of pentose sugars and 37 g/L of hexose sugars) into ethanol (Moniruzzaman *et al.*, (1998) *Biotechnol. Lett.* 20:943-947). It was noted that this strain was capable of fermenting a hemicellulose hydrolysate to completion within 48 to 72 hours, and under ideal conditions, within 24 hours.

Another preferred host cell of the invention is the bacterium *Klebsiella.* In particular, *Klebsiella oxytoca* is preferred because, like *E. coli,* this enteric bacterium has the native ability to metabolize monomeric sugars, which are the constituents of more complex sugars. Moreover, *K. oxytoca* has the added advantage of being able to transport and metabolize cellobiose and cellotriose, the soluble intermediates from the

enzymatic hydrolysis of cellulose (Lai *et al.*, (1996) *Appl. Environ. Microbiol.* 63:355-363; Moniruzzaman *et al.*, (1997) *Appl. Environ. Microbiol.* 63:4633-4637; Wood *et al.*, (1992) *Appl. Environ. Microbiol.* 58:2103-2110). The invention provides genetically engineered ethanologenic derivatives of *K. oxytoca, e.g.*, strain M5A1 having the *Z. mobilis pdc* and *adhB* genes encoded within the PET operon (as described herein and in U.S.P.N. 5,821,093; Wood *et al.*, (1992) *Appl. Environ. Microbiol.* 58:2103-2110).

Accordingly, the resulting organism, strain P2, produces ethanol efficiently from monomer sugars and from a variety of saccharides including raffinose, stachyose, sucrose, cellobiose, cellotriose, xylobiose, xylotriose, maltose, *etc.* (Burchhardt *et al.*, (1992) *Appl. Environ. Microbiol.* 58:1128-1133; Moniruzzaman *et al.*, (1997) *Appl. Environ. Microbiol.* 63:4633-4637; Moniruzzaman *et al.*, (1997) *J. Bacteriol.* 179:1880-1886; Wood *et al.*, (1992) *Appl. Environ. Microbiol.* 58:2103-2110). These strains may be further modified according to the methods of the invention to express and secrete a polysaccharase. Accordingly, this strain is suitable for use in the bioconversion of a complex saccharide in an SSF process (Doran *et al.*, (1993) *Biotechnol. Progress.* 9:533-538; Doran *et al.*, (1994) *Biotechnol. Bioeng.* 44:240-247; Wood *et al.*, (1992) *Appl. Environ. Microbiol.* 58:2103-2110). In particular, the use of this ethanologenic P2 strain eliminates the need to add supplemental cellobiase, and this is one of the least stable components of commercial fungal cellulases (Grohmann, (1994) *Biotechnol. Lett.* 16:281-286).

Screen for Promoters Suitable for Use in Heterologous Gene Expression

While in one embodiment, the surrogate promoter of the invention is used to improve the expression of a heterologous gene, *e.g.*, a polysaccharase, it will be appreciated that the invention also allows for the screening of surrogate promoters suitable for enhancing the expression of any desirable gene product. In general, the screening method makes use of the cloning vector described in Example 1 and depicted in Figure 3 that allows for candidate promoter fragments to be conveniently ligated and operably-linked to a reporter gene. In one embodiment, the *celZ* gene encoding glucanase serves as a convenient reporter gene because a strong colorimetric change results from the expression of this enzyme (glucanase) when cells bearing the plasmid are grown on a particular media (CMC plates). Accordingly, candidate promoters, *e.g.*,

a particular promoter sequence or, alternatively, random sequences that can be "shotgun" cloned and operably linked to the vector, can be introduced into a host cell and resultant colonies are scanned, visually, for having increased gene expression as evidenced by a phenotypic glucanase-mediated colorimetric change on a CMC plate. Colonies having the desired phenotype are then processed to yield the transforming DNA and the promoter is sequenced using appropriate primers (see Example 1 for more details).

The high correspondence between the glucanase-mediated colorimetric change on a CMC plate and expression levels of the enzyme is an excellent indication of the strength of a candidate promoter (Fig. 4). Hence, the methods of invention provide a rapid visual test for rating the strength of candidate surrogate promoters. Accordingly, depending on the desired expression level needed for a specific gene product, a particular identified surrogate promoter can be selected using this assay. For example, if simply the highest expression level is desired, then the candidate promoter that produces the largest colorimetric change may be selected. If a lower level of expression is desired, for example, because the intended product to be expressed is toxic at high levels or must be expressed at equivalent levels with another product, a weaker surrogate promoter can be identified, selected, and used as described.

III. Methods of Use

Degrading or Depolymerizing a Complex Saccharide

In one embodiment, the host cell of the invention is used to degrade or depolymerize a complex sugar *e.g.*, lignocellulose or an oligosaccharide into a smaller sugar moiety. To accomplish this, the host cell of the invention preferably expresses one or more polysaccharases, *e.g.*, a glucanase, and these polysaccharases may be liberated naturally from the producer organism. Alternatively, the polysaccharase is liberated from the producer cell by physically disrupting the cell. Various methods for mechanically (*e.g.*, shearing, sonication), enzymatically (*e.g.*, lysozyme), or chemically disrupting cells, are known in the art, and any of these methods may be employed. Once the desired polypeptide is liberated from the inner cell space it may be used to degrade a complex saccharide substrate into smaller sugar moieties for subsequent bioconversion into ethanol. The liberated cellulase may be purified using standard biochemical

techniques known in the art. Alternatively, the liberated polysaccharide need not be purified or isolated from the other cellular components and can be applied directly to the sugar substrate.

In another embodiment, a host cell is employed that coexpresses a polysaccharase and a secretory protein/s such that the polysaccharase is secreted into the growth medium. This eliminates the above-mentioned step of having to liberate the polysaccharase from the host cell. When employing this type of host, the host may be used directly in an aqueous solution containing a complex saccharide.

In another embodiment, a host cell of the invention is designed to express more than one polysaccharase or is mixed with another host expressing a different polysaccharase. For example, one host cell could express a heterologous β-glucosidase while another host cell could express an endoglucanase and yet another host cell could express an exoglucanase, and these cells could be combined to form a heterogeneous culture having multiple polysaccharase activities. Alternatively, in a preferred embodiment, a single host strain is engineered to produce all of the above polysaccharases. In either case, a culture of recombinant host/s is produced having high expression of the desired polysaccharases for application to a sugar substrate. If desired, this mixture can be combined with an additional cellulase, *e.g.*, an exogenous cellulase, such as a fungal cellulase. This mixture is then used to degrade a complex substrate. Alternatively, prior to the addition of the complex sugar substrate, the polysaccharase/s are purified from the cells and/or media using standard biochemical techniques and used as a pure enzyme source for depolymerizing a sugar substrate.

Finally, it will be appreciated by the skilled artisan, that the ethanol-producing bacterial strains of the invention are superior hosts for the production of recombinant proteins because, under anaerobic conditions (*e.g.*, in the absence of oxygen), there is less opportunity for improper folding of the protein (*e.g.*, due to inappropriate disulfide bond formation). Thus, the hosts and culture conditions of the invention potentially result in the greater recovery of a biologically active product.

Fermenting a Complex Saccharide

In a preferred embodiment of the present invention, the host cell having the above mentioned attributes is also ethanologenic. Accordingly, such a host cell can be

applied in degrading or depolymerizing a complex saccharide into a monosaccharide. Subsequently, the cell can catabolize the simpler sugar into ethanol by fermentation. This process of concurrent complex saccharide depolymerization into smaller sugar residues followed by fermentation is referred to as simultaneous saccharification and fermentation.

Typically, fermentation conditions are selected that provide an optimal pH and temperature for promoting the best growth kinetics of the producer host cell strain and catalytic conditions for the enzymes produced by the culture (Doran *et al.*, (1993) *Biotechnol. Progress.* 9:533-538). For example, for *Klebsiella*, *e.g.*, the P2 strain, optimal conditions were determined to be between 35-37° C and pH 5.0- pH 5.4. Under these conditions, even exogenously added fungal endoglucanases and exoglucanases are quite stable and continue to function for long periods of time. Other conditions are discussed in the Examples. Moreover, it will be appreciated by the skilled artisan, that only routine experimentation is needed, using techniques known in the art, for optimizing a given fermentation reaction of the invention.

Currently, the conversion of a complex saccharide such as lignocellulose, is a very involved, multi-step process. For example, the lignocellulose must first be degraded or depolymerized using acid hydrolysis. This is then followed by steps that separate liquids from solids and these products are subsequently washed and detoxified to result in cellulose and hemicellulose that can be further depolymerized (using added cellulases) and finally, fermented by a suitable ethanologenic host cell. In contrast, the fermenting of corn is much simpler in that amylases can be used to break down the corn starch for immediate bioconversion by an ethanologenic host in essentially a one-step process. Accordingly, it will be appreciated by the skilled artisan that the recombinant hosts and methods of the invention afford the use of a similarly simpler and more efficient process for fermenting lignocellulose. For example, the method of the invention is intended to encompass a method that avoids acid hydrolysis altogether. Moreover, the hosts of the invention have the following advantages, 1) efficiency of pentose and hexose co-fermentation; 2) resistance to toxins; 3) production of enzymes for complex saccharide depolymerization; and 4) environmental hardiness. Accordingly, the complexity of depolymerizing lignocellulose can be simplified using an improved biocatalyst of the invention. Indeed, in one preferred embodiment of the

invention, the reaction can be conducted in a single reaction vessel and in the absence of acid hydrolysis, *e.g.*. as an SSF process.

Potential Substrates for Bioconversion into Ethanol

One advantage of the invention is the ability to use a saccharide source that has been, heretofore, underutilized.

A number of complex saccharide substrates may be used as a starting source for depolymerization and subsequent fermentation using the host cells and methods of the invention. Ideally, a recyclable resource may be used in the SSF process. Mixed waste office paper is a preferred substrate (Brooks *et al.*, (1995) *Biotechnol. Progress.* 11:619-625; Ingram *et al.*, (1995) U.S.P.N. 5,424,202), and is much more readily digested than acid pretreated bagasse (Doran *et al.*, (1994) *Biotech. Bioeng.* 44:240-247) or highly purified crystalline cellulose (Doran *et al.* (1993) *Biotechnol. Progress.* 9:533-538). Since glucanases, both endoglucanases and exoglucanases, contain a cellulose binding domain, and these enzymes can be readily recycled for subsequent fermentations by harvesting the undigested cellulose residue using centrifugation (Brooks *et al.*, (1995) *Biotechnol. Progress.* 11:619-625). By adding this residue with bound enzyme as a starter, ethanol yields (per unit substrate) were increased to over 80% of the theoretical yield with a concurrent 60% reduction in fungal enzyme usage (Figure 2). Such approaches work well with purified cellulose, although the number of recycling steps may be limited with substrates with a higher lignin content. Other substrate sources that are within the scope of the invention include any type of processed or unprocessed plant material, *e.g.*, lawn clippings, husks, cobs, stems, leaves, fibers, pulp, hemp, sawdust, newspapers, *etc.*

This invention is further illustrated by the following examples which should not be construed as limiting.

EXAMPLE 1

Methods for Making Recombinant *Escherichia* Hosts Suitable for Fermenting Oligosaccharides into Ethanol

In this example, methods for developing and using *Escherichia* hosts suitable for fermenting oligosaccharides into ethanol are described. In particular, a strong promoter

is identified which can be used to increase the expression of a polysaccharase (*e.g.*, glucanase). In addition, genes from *Erwinia chrysanthemi* are employed to facilitate polysaccharase secretion thereby eliminating the need for cell disruption in order to release the desired polysaccharase activity.

Throughout this example, the following materials and methods are used unless otherwise stated.

Materials and Methods

Organisms and Culture Conditions

The bacterial strains and plasmids used in this example are listed in Table 1, below.

For plasmid constructions, the host cell *E. coli* DH5α was used. The particular gene employed encoding a polysaccharase (*e.g.*, glucanase) was the *celZ* gene derived from *Erwinia chrysanthemi* P86021 (Beall, (1995) Ph.D. Dissertation, University of Florida; Wood *et al.*, (1997) *Biotech. Bioeng.* 55:547-555). The particular genes used for improving secretion were the *out* genes derived from *E. chrysanthemi* EC16 (He *et al.*, (1991) *Proc. Natl. Acad. Sci. USA.* 88:1079-1083).

Typically, host cell cultures were grown in Luria-Bertani broth (LB) (10 g L^{-1} Difco® tryptone, 5 g L^{-1} Difco® yeast extract, 5 g L^{-1} sodium chloride) or on Luria agar (LB supplemented with 15 g L^{-1} of agar). For screening host cells having glucanase *celZ* activity (EGZ), CMC-plates (Luria agar plates containing carboxymethyl cellulose (3 g L^{-1})) were used (Wood *et al.*, (1988) *Methods in Enzymology* 160:87-112). When appropriate, the antibiotics ampicillin (50 mg L^{-1}), spectinomycin (100 g L^{-1}), kanamycin (50 g L^{-1}) were added to the media for selection of recombinant or integrant host cells containing resistance markers. Constructs containing plasmids with a temperature conditional pSC101 replicon (Posfai *et al.*, (1997) *J. Bacteriol.* 179:4426-4428) were grown at 30°C and, unless stated otherwise, constructs with pUC-based plasmids were grown at 37°C.

TABLE 1. Strains and Plasmids Used

Strains/Plasmids	Description	Sources/References
Strains		
Z. mobilis CP4	Prototrophic	Osman *et al.*, (1985) *J. Bact.* 164:173-180
E. coli strain DH5α	*lacZ M15 recA*	Bethesda Research Laboratory
E. coli strain B	prototrophic	ATCC 11303
E. coli strain HB 101	*recA lacY recA*	ATCC 37159
Plasmids		
pUC19	*bla* cloning vector	New England Biolabs
pST76-K	*kan* low copy number, temp. sens.	
pRK2013	*kan* mobilizing helper plasmid (*mob*−)	ATCC
pCPP2006	Sp^r, ca. 40 kbp plasmid carrying the complete *out* genes from *E. chrysanthemi* EC16	He *et al.*, (1991) *P.N.A.S.* 88:1079-1083
pLOI1620	*bla celZ*	Beall *et al*, (1995) Ph.D. Dissertation, U. of Florida
pLOI2164	pLOI1620 with *Bam*HI site removed (Klenow)	See text
pLOI2170	*Nde*I-*Hind*III fragment (promoterless *celZ*) from pLOI2164 cloned into pUC19	See text
pLOI2171	*Bam*HI-*Sph*I fragment (promoterless *celZ*) from pLOI2170 cloned into pST76-K	See text
pLOI2173	*Eco*RI-*Sph*I fragment (*celZ* with native promoter) from pLOI2164 cloned into pST76-K	See text
pLOI2174	*Eco*RI-*Bam*HI fragment (*gap* promoter) cloned into pLOI2171	See text
pLOI2175	*Eco*RI-*Bam*HI fragment (*eno* promoter) cloned into pLOI2171	See text
pLOI2177	Random *Sau*3A1 *Z. mobilis* DNA fragment cloned into pLOI2171	See text
pLOI2178	Random *Sau*3A1 *Z. mobilis* DNA fragment cloned into pLOI2171	See text
pLOI2179	Random *Sau*3A1 *Z. mobilis* DNA fragment cloned into pLOI2171	See text
pLOI2180	Random *Sau*3A1 *Z. mobilis* DNA fragment cloned into pLOI2171	See text

pLOI2181	Random *Sau*3A1 *Z. mobilis* DNA fragment cloned into pLOI2171	See text
pLOI2182	Random *Sau*3A1 *Z. mobilis* DNA fragment cloned into pLOI2171	See text
pLOI2183	Random *Sau*3A1 *Z. mobilis* DNA fragment cloned into pLOI2171	See text
pLOI2184	Random *Sau*3A1 *Z. mobilis* DNA fragment cloned into pLOI2171	See text
pLOI2196	pLOI2177 fused into pUC19 at the *Pst*I site	See text
pLOI2197	pLOI2180 fused into pUC19 at the *Pst*I site	See text
pLOI2198	pLOI2182 fused into pUC19 at the *Pst*I site	See text
pLOI2199	pLOI2183 fused into pUC19 at the *Pst*I site	See text
pLOI2307	*Eco*RI-*Sph*I fragment from pLOI2183 cloned into pUC19	See text

Genetic Methods

Standard techniques were used for all plasmid constructions (Ausubel *et al.*, (1987) *Current Protocols in Molecular Biology*, John Wiley & Sons, Inc.; Sambrook *et al.*, (1989) *Molecular cloning: a laboratory manual*, 2nd ed. C.S.H.L., Cold Spring Harbor, N.Y). For conducting small-scale plasmid isolation, the TELT procedure was performed. For large-scale plasmid isolation, the Promega® Wizard Kit was used. For isolating DNA fragments from gels, the Qiaquick® Gel Extraction Kit from Qiagen® was employed. To isolate chromosomal DNA from *E. coli* and *Z. mobilis* the methods of Cutting and Yomano were used (Cutting *et al.*, (1990). Genetic analysis, pp. 61-74. In, Molecular biological methods for *Bacillus*. John Wiley & Sons, Inc.; Yomano *et al.*, (1993) *J. Bacteriol.* 175:3926-3933).

To isolate the two glycolytic gene promoters (*e.g.*, *gap* and *eno*) described herein, purified chromosomal DNA from *E. coli* DH5α was used as a template for the PCR (polymerase chain reaction) amplification of these nucleic acids using the following primer pairs: *gap* promoter, 5′-CGAATTCCTGCCGAAGTTTATTAGCCA-3' (SEQ ID NO: 3) and 5′-AAGGATCCTTCCACCAGCTATTTGTTAGTGA-3′ (SEQ ID NO: 4); *eno* promoter, 5′-AGAATTCTGCCAGTTGGTTGACGATAG-3' (SEQ ID NO: 5) and 5′-CAGGATCCCCTCAAGTCACTAGTTAAACTG-3' (SEQ ID NO: 6). The *out* genes encoding secretory proteins derived from *E. chrysanthemi* (pCPP2006) were

conjugated into *E. coli* using pRK2013 for mobilization (Figurski *et al.*, (1979) *Proc. Natl. Acad. Sci. USA.* 76: 1648-1652; Murata *et al.*, (1990) *J. Bacteriol.* 172:2970-2978).

To determine the sequence of various DNAs of interest, the dideoxy sequencing method using fluorescent primers was performed on a LI-COR Model 4000-L DNA Sequencer. The pST76-K-based plasmids were sequenced in one direction using a T7 primer (5'-TAATACGACTCACTATAGGG-3' (SEQ ID NO: 7)). The pUC18- and pUCI9-based plasmids were sequenced in two directions using either a forward primer (5'-CACGACGTTGTAAAACGAC-3' (SEQ ID NO: 8)) or a reverse primer (5'-TAACAATTTCACACAGGA-3' (SEQ ID NO: 9)). The extension reactions of the sequencing method were performed using a Perkin Elmer GeneAmp® PCR 9600 and SequiTherm Long-Read Sequencing Kit-LC®. Resultant sequences were subsequently analyzed using the Wisconsin Genetic Computer Group (GCG) software package (Devereux *et al.*, (1984) *Nucleic Acids Rev.* 12:387-395).

To determine the start of transcriptional initiation in the above-mentioned promoters, primer extension analysis was performed using standard techniques. In particular, promoter regions were identified by mapping the transcriptional start sites using a primer finding correspondence within the *celZ* gene RNA that was isolated from cells in late exponential phase using a Qiagen RNeasy® kit. Briefly, cells were treated with lysozyme (400 µg/ml) in TE (Tris-HCl, EDTA) containing 0.2 M sucrose and incubated at 25° C for 5 min prior to lysis. Liberated RNA was subjected to ethanol precipitation and subsequently dissolved in 20 µl of Promega® AMV reverse transcriptase buffer (50 mM Tris-HCl, pH 8.3, 50 mM KCl, 10 mM $MgCl_2$, 0.5 mM spermadine, 10 mM DTT). An IRD41-labeled primer (5'-GACTGGATGGTTATCCGAATAAGAGAGAGG-3' (SEQ ID NO: 10)) from LI-Cor Inc. was then added and the sample was denatured at 80° C for 5 min, annealed at 55° C for 1 hr, and purified by alcohol precipitation. Annealed samples were dissolved in 19 µl of AMV reverse transcriptase buffer containing 500 µM dNTPs and 10 units AMV reverse transcriptase, and incubated for extension (1 h at 42°C). Products were treated with 0.5 µg/ml DNase-free RNase A, precipitated, dissolved in loading buffer, and compared to parallel dideoxy promoter sequences obtained using the LI-COR Model 4000-L DNA sequencer.

Polysaccharase Activity

To determine the amount of polysaccharase activity (*e.g.*, glucanase activity) resulting from expression of the *celZ* gene, a Congo Red procedure was used (Wood *et al.*, (1988) *Methods in Enzymology* 160:87-112). In particular, selected clones were transferred to gridded CMC plates and incubated for 18 h at 30° C and then stained and recombinant host cells expressing glucanase formed yellow zones on a red background. Accordingly, the diameters of these colorimetric zones were recorded as a relative measure of *celZ* expression.

Glucanase activity (EGZ) was also measured using carboxymethyl cellulose as a substrate. In this test, appropriate dilutions of cell-free culture broth (extracellular activity) or broth containing cells treated with ultrasound (total activity) were assayed at 35° C in 50 mM citrate buffer (pH 5.2) containing carboxymethyl cellulose (20 g L^{-1}). Conditions for optimal enzyme release for 3-4 ml samples were determined to be 4 pulses at full power for 1 second each using a cell disruptor (Model W-220F, Heat System-Ultrasonics Inc., Plainview, NY). To stop the enzyme reactions of the assay, samples were heated in a boiling water bath for 10 min. To measure reducing sugars liberated enzymatically by the glucanase, a dinitrosalicylic acid reagent was employed using glucose as a standard (Wood *et al.*, (1988) *Methods in Enzymology* 160:87-112). The amount of enzyme activity (IU) was expressed as μmols of reducing sugar released per min or as a percentage of total activity from an average of two or more determinations.

Ultrastructural Analysis

To determine the ultrastructure of various recombinant host cells, fresh colonies from Luria agar plates were prepared for analysis by fixing in 2% glutaraldehyde in 0.2 M sodium cacodylate buffer (pH 7) followed by incubation in 1% osmium tetroxide and followed by 1% uranyl acetate in distilled water. Samples were dehydrated in ethanol, embedded in Spurr's plastic, and ultrathin sections were prepared and examined using a Zeiss® EM-IOCA electron microscope (Spur (1969) *J. Ultrastruct. Res.* 26:31).

Construction of a Low Copy Promoter Probe Vector Using celZ as the Reporter Gene

To facilitate the isolation of strong promoters, a low copy vector was constructed with a pSC101 replicon and a *Bam*HI site immediately preceding a promoterless *celZ* gene (pL012171). Accordingly, this promoterless plasmid was used as a negative control. The plasmid pLOI1620 was used as a source of *celZ* and is a pUC18 derivative with expression from consecutive *lac* and *celZ* promoters. The *Bam*HI site in this plasmid was eliminated by digestion and Klenow treatment (pLOI2164). The *celZ* gene was isolated as a promoterless *Nde*I fragment after Klenow treatment. The resulting blunt fragment was digested with *Hind*III to remove downstream DNA and ligated into pUC19 (*Hind*III to *Hinc*II) to produce pLOI2170. In this plasmid, *celZ is* oriented opposite to the direction of *lacZ* transcription and was only weakly expressed. The *Bam*HI (amino terminus)-*Sph*I (carboxyl terminus) fragment from pLOI2170 containing *celZ* was then cloned into the corresponding sites of pST76-K, a low copy vector with a temperature sensitive replicon, to produce pLOI2171 (Fig. 3). Expression of *celZ* in this vector was extremely low facilitating its use as a probe for candidate strong promoters.

Analysis of celZ Expression from Two E. coli Glycolytic Promoters (gap and eno)

Two exemplary promoters driving glycolytic genes (*gap* and *eno*) in *E. coli* were examined for their ability to drive the expression of the heterologous *celZ* gene encoding glucanase. Chromosomal DNA from the *E. coli* DH5α strain was used as a template to amplify the *gap* and *eno* promoter regions by the polymerase chain reaction. The resulting fragments of approximately 400 bp each were digested with *Eco*RI and *Bam*HI and cloned into the corresponding sites in front of a promoterless *celZ* gene in pLOI2171 to produce pLOI2174 *(gap* promoter) and pLOI2175 *(eno* promoter). As a control, the *Eco*RI-*Sph*I fragment from pLOI2164 containing the complete *celZ* gene and native *E. chrysanthemi* promoter was cloned into the corresponding sites of pST76-K to produce pLOI2173. These three plasmids were transformed into *E. coli* strains B and DH5α and glucanase activity (EGZ) was compared. For both strains of *E. coli,* glucanase activities were lower on CMC plates with *E. coli* glycolytic promoters than with pLOI2173 containing the native *E. chrysanthemi* promoter (Table 2). Assuming activity is related to the square of the radius of each zone (Fick's Law of diffusion), EGZ production with glycolytic promoters (pLOI2174 and pLOI2175) was

estimated to be 33% to 65% lower than in the original construct. Accordingly, other candidate promoters for driving high levels of *celZ* gene expression were investigated.

Identifying and Cloning Random DNA Fragments Suitable for Use as Promoters for Heterologous Gene Expression

Random fragments derived from *Z. mobilis* can be an effective source of surrogate promoters for the high level expression of heterologous genes in *E. coli.* (Conway *et al.*, (1987) *J. Bacteriol.* 169:2327-2335; Ingram *et al.*, (1988) *Appl. Environ. Micro.* 54:397-404). Accordingly, to identify surrogate promoters for *Erwinia celZ* expression, *Z. mobilis* chromosomal DNA was extensively digested with *Sau*3AI and resulting fragments were ligated into pLOI2171 at the *Bam*HI site and transformed into *E. coli* DH5α to generate a library of potential candidate promoters. To rapidly identify superior candidate promoters capable of driving *celZ* gene expression in *E. coli*, the following biological screen was employed. Colonies transformed with *celZ* plasmids having different random candidate promoters were transferred to gridded CMC plates and stained for glucanase activity after incubation (Table 2). Approximately 20% of the 18,000 clones tested were CMC positive. The 75 clones which produced larger zones than the control, pLOI2173, were examined further using another strain, *E. coli* B.

TABLE 2. Evaluation of promoter strength for *celZ* expression in *E. coli* using CMC indicator plates.

	E. coli DH5α host			*E. coli* B host		
Plasmids	Number of Plasmids[a]	CMC zone diameter (mm)[b]	% of native promoter ($100*R^2_x/R^2_c$)[c]	Number of plasmids	CMC zone diameter (mm)	% of native promoter ($100*R^2_x/R^2_c$)
pLOI2171 (promoterless)	1	0	--	--	--	--
pLOI2173 (native promoter)	1	5.0	100	1	4.5	100
pLOI2174 (*gap* promoter)	1	4.0	77	1	3.5	60
pLOI2175	1	3.0	43	1	2.8	35

(*eno* promoter)						
Z. mobilis promoters						
Group I	5	13.0	676	4	10.8-11.3	570-625
Group II	14	9.0-11.0	324-484	17	9.0-10.5	445-545
Group III	56	6.0-9.0	144-324	54	5.0-8.8	125-375

[a] The number of clones which the indicated range of activities.

[b] The average size of the diameters from three CMC digestion zones.

[c] R^2_X is the square of the radius of the clear zone with the test plasmid: R^2_C is the square of the radius of the clear zone for the control (pLOI2173).

Thus, promoter strength for selected candidate promoters was confirmed in two different strains with, in general, recombinants of DH5α producing larger zones (*e.g.*, more glucanase) than recombinants of strain B. However, relative promoter strength in each host was similar for most clones. Based on these analyses of glucanase production as measured by zone size using CMC plates, four clones appeared to express *celZ* at approximately 6-fold higher levels than the construct with the original *E. chrysanthemi celZ* gene (pLOI2173), and at 10-fold higher levels than either of the *E. coli* glycolytic promoters. Accordingly, these and similarly strong candidate promoters were selected for further study.

Production and Secretion of Glucanase

Eight plasmid derivatives of pST76-K (pLOI2177 to pLOI2184) were selected from the above-described screen (see Group I and Group II (Table 2)) and assayed for total glucanase activity in *E. coli* strain B (Table 3). The four plasmids giving rise to the largest zones on CMC plates were also confirmed to have the highest glucanase activities (pLOI2177, pLOI2180, pLOI2182, and pLOI2183). The activities were approximately 6-fold higher than that of the unmodified *celZ* (pLOI2173), in excellent agreement with our estimate using the square of the radius of the cleared zone on CMC plates. Figure 4 shows a comparison of activity estimates from CMC plates and *in vitro* enzyme assays for strain B containing a variety of different promoters, with and without the addition of *out* genes encoding secretory proteins. Although there is some scatter, a

direct relationship is clearly evident which validates the plate method for estimating relative activity. The original construct in pUC18, a high copy plasmid, was also included for comparison (pLOI2164). This construct with consecutive *lac* and *celZ* promoters produced less EGZ activity than three of the low copy plasmids with surrogate promoters (pLOI2177, pLOI2182, and pLOI2183). Thus, to increase *celZ* expression of glucanase even more, the DNA fragment containing *celZ* and the most effective surrogate promoter was isolated from pLOI2183 (as a *Eco*RI-*Sph*I fragment) and inserted into pUC19 with transcription oriented opposite to that of the *lac* promoter (pLOI2307). Accordingly, the above-identified strong surrogate promoter when incorporated into a high copy plasmid, further increased glucanase activity by 2-fold.

Engineering Increased Secretion of Glucanase

To further improve on the above-described results for increasing expression of *celZ* encoded glucanase, the above host cells were engineered for increased secretion. Genes encoding secretory proteins (*e.g.*, the *out* genes) derived from *E. chrysanthemi* EC16 were used for improving the export of the glucanase using the plasmid as described in He *et al.* that contains *out* genes (pCPP2006) (He *et al.*, (1991) *Proc. Natl. Acad. Sci. USA.* 88:1079-1083). The increased secretion of EGZ in *E. coli* B was investigated and results are presented in Table 3.

TABLE 3. Comparison of promoters for EGZ production and secretion in *E. coli B*

Plasmids[a]	Without secretion genes		With secretion genes (pCPP2006)	
	Total activity (IU/L)[b]	Extracellular[c](%)	Total Activity (IU/L)	Extracellular[c] (%)
pLOI2173	620	17	1,100	43
pLOI2177	3,700	10	5,500	44
pLOI2178	2,200	9	3,500	49
pLOI2179	2,000	10	3,000	50
pLOI2180	2,900	8	6,300	39
pLOI2181	1,800	11	4,100	46
pLOI2182	3,500	7	6,600	38
pLOI2183	3,400	7	6,900	39
pLOI2184	2,100	12	2,400	39
pLOI2164	3,200	20	6,900	74
pLOI2307	6,600	28	13,000	60

[a] Plasmids pLOI2173 and pLOI2164 contain the *celZ* native promoter; pLOI2307 contains the strong promoter from pLOI2183.

Plasmids pLOI2164 and pLOI2307 are pUC-based plasmids (high copy number). All other plasmids are derivatives of pST76-K (low copy number).

[b] Glucanase activities were determined after 16 h of growth at 30°C.

[c] Extracellular activity (secreted or released).

Recombinant hosts with low copy plasmids produced only 7- 17% of the total EGZ extracellularly (after 16 h of growth) without the additional heterologous secretory proteins (*out* proteins encoded by plasmid pCPP2006). A larger fraction of EGZ (20-28%) was found in the extracellular broth surrounding host cells with the high-copy pUC-based plasmids than with the low copy pST76-based plasmids containing the same promoters. However, in either case, the addition of *out* genes encoding secretory proteins (*e.g.*, pCPP2006) increased the total level of expression by up to 2-fold and

increased the fraction of extracellular enzyme (38-74%) by approximately 4-fold. The highest activity, 13,000 IU/L of total glucanase of which 7,800 IU/L was found in the cell-free supernatant was produced by strain B having both pLOI2307 encoding *celZ* driven by a strong surrogate promoter and pCPP2006 encoding *out* secretory proteins).

It has been reported that under certain conditions (pH 7, 37° C), the specific activity for pure EGZ enzyme is 419 IU (Py *et al.*, (1991) *Protein Engineering* 4:325-333) and it has been determined that EGZ produced under these conditions is 25% more active than under the above-mentioned conditions (pH 5.2 citrate buffer, 35° C). Accordingly, assuming a specific activity of 316 IU for pure enzyme at pH 5.2 (35°C), the cultures of *E. coli* B (containing pLOI2307 and pCPP2006, *e.g.*, plasmids encoding glucanase and secretory proteins), produced approximately 41 mg of active EGZ per liter or 4-6% of the total host cell protein was active glucanase.

Sequence Analysis of the Strongest Promoter Derived from Z. mobilis

The sequences of the four strongest surrogate promoters (pLOI2177, pLOI2180, pLOI2182, and pLOI2183) were determined. To facilitate this process, each was fused with pUC19 at the *Pst*I site. The resulting plasmids, pLOI2196, pLOI2197, pLOI2198, and pLOI2199, were produced at high copy numbers (ColEI replicon) and could be sequenced in both directions using M13 and T7 sequencing primers. All four plasmids contained identical pieces of *Z. mobilis* DNA and were siblings. Each was 1417 bp in length and contained 4 internal Sau3AI sites. DNA and translated protein sequences (six reading frames) of each piece were compared to the current data base. Only one fragment (281 bp internal fragment) exhibited a strong match in a Blast search (National Center for Biotechnology Information; http://www.ncbi.nlm.nih.gov/BLAST/) and this fragment was 99% identical in DNA sequence to part of the *Z. mobilis hpnB* gene which is proposed to function in cell envelope biosynthesis (Reipen *et al.*, (1995) *Microbiology* 141:155-161). Primer extension analysis revealed a single major start site, 67 bp upstream from the *Sau*3AI/*Bam*HI junction site with *celZ,* and a second minor start site further upstream (Fig. 5). Sequences in the -10 and -35 regions were compared to the conserved sequences for *E. coli* sigma factors (Wang *et al.*, (1989) *J. Bacteriol.* 180:5626-5631; Wise *et al.*, (1996) *J. Bacteriol.* 178:2785-2793). The dominant

promoter region (approximately 85% of total start site) appears similar to a sigma^{70} promoter while the secondary promoter site resembles a sigma^{38} promoter.

Microscopic Analysis of Recombinant Host Cells Producing Glucanase

Little difference in cell morphology was observed between recombinants and the parental organism by light microscopy. Under the electron microscope, however, small polar inclusion bodies were clearly evident in the periplasm of strain B (pLOI2164) expressing high amounts of glucanase and these inclusion bodies were presumed to contain EGZ (Fig. 6). In the strain B (pLOI2307) that produced 2-fold higher glucanase activity the inclusion bodies were even larger and occupied up to 20% of the total cell volume. The large size of these polar bodies suggests that glucanase activity measurements may underestimate the total EGZ production. Typically, polar inclusion bodies were smaller in host cells also having constructs encoding the *out* secretory proteins which allow for increased secretion of proteins from the periplasmic space. As expected, no periplasmic inclusion bodies were evident in the negative control strain B (pUC19) which does not produce glucanase.

EXAMPLE 2

Recombinant *Klebsiella* Hosts Suitable for Fermenting Oligosaccharides into Ethanol

In this example, a recombinant *Klebsiella* host, suitable for use as a biocatalyst for depolymerizing and fermenting oligosaccharides into ethanol, is described.

Materials and Methods Used in this Example

Unless otherwise stated, the following materials and methods were used in the example that follows.

Bacteria, Plasmids, and Culture Conditions

The strains and plasmids that were used in this exemplification are summarized in Table 4 below.

TABLE 4. Strains and Plasmids Used

Strains/Plasmids	Properties	Sources/References
Strains		
Zymomonas mobilis CP4	prototrophic	Ingram *et al.* (1988) *Appl. Environ. Micro.* 54:397-404
Escherichia coli		
DH5α	*lacZ* M15 *recA*	Bethesda Research Laboratory
HB101	*recA lacY recA*	ATCC 37159
Klebsiella oxytoca		
M5A1	prototrophic	Wood *et al.* (1992) *Appl. Environ. Micro.* 58:2103-2110
P2	*Pfl::pdc adhB cat*	Wood *et al.* (1992) *Appl. Environ. Micro.* 58:2103-2110
SZ1	*pfl::pdc adhB cat*; integrated *celZ*; *tet*	See text
SZ2	*pfl::pdc adhB cat*; integrated *celZ*; *tet*	See text
SZ3	*pfl::pdc adhB cat*; integrated *celZ*; *tet*	See text
SZ4	*pfl::pdc adhB cat*; integrated *celZ*; *tet*	See text
SZ5	*pfl::pdc adhB cat*; integrated *celZ*; *tet*	See text
SZ6	*pfl::pdc adhB cat*; integrated *celZ*; *tet*	See text
SZ7	*pfl::pdc adhB cat*; integrated *celZ*; *tet*	See text
SZ8	*pfl::pdc adhB cat*; integrated *celZ*; *tet*	See text
SZ9	*pfl::pdc adhB cat*; integrated *celZ*; *tet*	See text
SZ10	*pfl::pdc adhB cat*; integrated *celZ*; *tet*	See text
Plasmids		
pUC19	*bla* cloning vector	New England Biolab
pBR322	*bla tet* cloning vector	New England Biolab
pLOI1620	*bla celZ*	Wood *et al.* (1997) *Biotech. Bioeng. 55:547-555*
pRK2013	*kan* mobilizing helper plasmid (mob^-)	ATCC
pCPP2006	Sp^r, 40 kbp fragment containing *out* genes from *E. chrysanthemi* EC16	He *et al.* (1991) *P.N.A.S.* 88:1079-1083
pST76-K	*kan* low copy vector containing temperature sensitive pSC101 replicon	Posfai *et al.* (1997) *J. Bact.* 179:4426-4428
pLOI2164	*bla celZ (Bam*HI eliminated from pLOI1620)	See text
pLOI2173	*kan celZ* (native *celZ* promoter)	See text

pLOI2177	*kan celZ* (surrogate promoter from *Z. mobilis*)	See text
pLOI2178	*kan celZ* (surrogate promoter from *Z. mobilis*)	See text
pLOI2179	*kan celZ* (surrogate promoter from *Z. mobilis*)	See text
pLOI2180	*kan celZ* (surrogate promoter from *Z. mobilis*)	See text
pLOI2181	*kan celZ* (surrogate promoter from *Z. mobilis*)	See text
pLOI2182	*kan celZ* (surrogate promoter from *Z. mobilis*)	See text
pLOI2183	*kan celZ* (surrogate promoter from *Z. mobilis*)	See text
pLOI2184	*kan celZ* (surrogate promoter from *Z. mobilis*)	See text

TABLE 4. Strains and Plasmids Used (*continued*)

Strains/Plasmids	Properties	Sources/References
pLOI2185	*kan celZ* (surrogate promoter from *Z. mobilis*)	See text
pLOI2186	*kan celZ* (surrogate promoter from *Z. mobilis*)	See text
pLOI2187	*kan celZ* (surrogate promoter from *Z. mobilis*)	See text
pLOI2188	*kan celZ* (surrogate promoter from *Z. mobilis*)	See text
pLOI2189	*kan celZ* (surrogate promoter from *Z. mobilis*)	See text
pLOI2190	*kan celZ* (surrogate promoter from *Z. mobilis*)	See text
pLOI2191	*kan celZ* (surrogate promoter from *Z. mobilis*)	See text
pLOI2192	*kan celZ* (surrogate promoter from *Z. mobilis*)	See text
pLOI2193	*kan celZ* (surrogate promoter from *Z. mobilis*)	See text
pLOI2194	*kan celZ* (surrogate promoter from *Z. mobilis*)	See text
pLOI2301	*Asc*I linker inserted into *Nde*I site of pUC19	See text
pLOI2302	*Asc*I linker inserted into *Sap*I site of pLOI2301	See text
pLOI2303	*Ava*I-*Eco*RI fragment from pBR322 inserted into *Pst*I site of pLOI2302 after Klenow treatment	See text
pLOI2305	*Eco*RI DNA fragment of *K. oxytoca* M5A1 genomic DNA (ca. 2.5 kb) cloned into the *Sma*I site of pLOI2303	See text
pLOI2306	*Eco*RI-*Sph*I fragment from pLOI2183 cloned into *Eco*RI site of pLOI2305	See text

The culture conditions used for cultivating *E. coli* and *K. oxytoca* M5A1 typically employed Luria-Bertani broth (LB) containing per liter: 10 g Difco® tryptone, 5 g yeast extract, and 5 g sodium chloride, or, alternatively, Luria agar (LB supplemented with 15 g of agar) (Sambrook *et al.*, (1989), *Molecular Cloning: A Laboratory Manual*, C.S.H.L., Cold Spring Harbor, N.Y.).

For screening bacterial colonies under selective conditions, CMC-plates (Luria agar plates containing 3 g L^{-1} carboxymethyl cellulose) were used to determine levels of glucanase activity expressed by a given bacterial strain (Wood *et al.* (1988) *Enzymology*, 160:87-112). For cultivating ethanologenic strains, glucose was added to solid media (20 g L^{-1}) and broth (50 g L^{-1}). In determining glucanase activity, the glucose in the growth media was replaced with sorbitol (50 g L^{-1}), a non-reducing sugar. For cultivating various strains or cultures in preparation for introducing nucleic acids by electroporation, a modified SOC medium was used (*e.g.*, 20 g L^{-1} Difco® tryptone, 5 g L^{-1} , Difco® yeast extract, 10 mM NaCl, 2.5 mM KCl, 10 mM $MgSO_4$, 10 mM $MgCl_2$, and 50 g L^{-1} glucose). The antibiotics ampicillin (50 mg L^{-1}), spectinomycin (100 mg L^{-1}), kanamycin (50 mg L^{-1}), tetracycline (6 or 12 mg L^{-1}), and chloramphenicol (40, 200, or 600 mg L^{-1}) were added when appropriate for selection of recombinant hosts bearing antibiotic resistance markers. Unless stated otherwise, cultures were grown at 37° C. Ethanologenic strains and strains containing plasmids with a temperature-sensitive pSC101 replicon were grown at 30° C.

Genetic Methods

For plasmid construction, cloning, and transformations, standard methods and *E. coli* DH5α hosts were used (Ausubel *et al.* (1987) Current Protocols in Molecular Biology. John Wiley & Sons, Inc.; Sambrook *et al.*, (1989) *Molecular Cloning: A Laboratory Manual*, C.S.H.L., Cold Spring Harbor, N.Y.). Construction of the *celZ* integration vector, pLOI2306, was performed as shown in Figure 7. A circular DNA fragment lacking a replicon from pLOI2306 (see Figure 7) was electroporated into the ethanologenic *K. oxytoca* P2 using a Bio-Rad Gene Pulser using the following conditions: 2.5 kV and 25 μF with a measured time constant of 3.8-4.0 msec (Comaduran *et al.* (1998) *Biotechnol. Lett.* 20:489-493). The *E. chrysanthemi* EC 16 secretion system (pCPP2006) was conjugated into *K. oxytoca* using pRK2013 for

mobilization (Murata *et al.* (1990) *J. Bacteriol.* 172:2970-2978). Small scale and large scale plasmid isolations were performed using the TELT procedure and a Promega Wizard Kit, respectively. DNA fragments were isolated from gels using a Qiaquick® Gel Extraction Kit from Qiagen® (Qiagen Inc., Chatsworth, CA). Chromosomal DNA from *K. oxytoca* M5A1 and *Z. mobilis* CP4 were isolated as described by Cutting and Yomano (see Example 1). The DNAs of interest were sequenced using a LI-COR Model 4000-L DNA sequencer (Wood *et al.* (1997) *Biotech. Bioeng.* 55:547-555).

Chromosomal Integration of celZ

Two approaches were employed for chromosomal integration of *celZ*, using selection with a temperature-conditional plasmid (pLOI2183) using a procedure previously described for *E. coli* (Hamilton *et al.*, (1989) *J. Bacteriol.* 171:4617-4622) and direct integration of circular DNA fragments lacking a functional replicon. This same method was employed for chromosomal integration of *Z. mobilis* genes encoding the ethanol pathway in *E. coli* B (Ohta K *et al.*, (1991) *Appl. Environ. Microbiol.* 57:893-900) and *K. oxytoca* M5A1 (Wood *et al.* (1992) *Appl. Environ. Microbiol.* 58:2103-2110). Typically, circular DNA was transformed into P2 by electroporation using a Bio-Rad Gene Pulser. Next, transformants were selected on solid medium containing tetracycline (6 mg L^{-1}) and grown on CMC plates to determine levels of glucanase activity.

Glucanase Activity

Glucanase activity resulting from expression of *celZ* gene product (*i.e.*, glucanase) under the control of different test promoters was evaluated by staining CMC plates as described in Example 1. This colorimetric assay results in yellow zones indicating glucanase activity and the diameter of the zone was used as a relative measure of *celZ* polypeptide expression. Clones that exhibited the largest zones of yellow color were further evaluated for glucanase activity at 35° C using carboxymethyl cellulose as the substrate (20 g L^{-1} dissolved in 50 mM citrate buffer, pH 5.2) (Wood *et al.* (1988) *Methods in Enzymology* 160: 87-112). In order to measure the amount of intracellular glucanase, enzymatic activity was released from cultures by treatment with ultra-sound for 4 seconds (Model W-290F cell disruptor, Heat System-Ultrasonics Inc., Plainview,

NY). The amount of glucanase activity expressed was measured and is presented here as μmol of reducing sugar released per min (IU). Reducing sugar was measured as described by Wood (Wood *et al.* (1988) *Methods in Enzymology* 160: 87-112) using a glucose standard.

Substrate Depolymerization

To further determine the amount of glucanase activity produced by various host cells, different carbohydrate substrates were incubated with various cell extracts (20 g L^{-1} suspended in 50 mM citrate buffer, pH 5.2). In one example, test substrates comprising acid-swollen and ball-milled cellulose were prepared as described by Wood (Wood *et al.* (1988) *Methods in Enzymology* 160: 87-112). A typical polysaccharase extract (*i.e.*, EGZ (glucanase) from *K. oxytoca* SZ6 (pCPP2006)) was prepared by cultivating the host cells at 30°C for 16 h in LB supplemented with sorbitol, a nonreducing sugar. Dilutions of cell-free broth were added to substrates and incubated at 35°C for 16 h. Several drops of chloroform were added to prevent the growth of adventitious contaminants during incubation. Samples were removed before and after incubation to measure reducing sugars by the DNS method (see, Wood *et al.* (1988) *Methods in Enzymology* 160: 87-112). The degree of polymerization (DP) was estimated by dividing the total calculated sugar residues present in the polymer by the number of reducing ends.

Fermentation Conditions

Fermentations were carried out in 250 ml flasks containing 100 ml of Luria broth supplemented with 50 g L^{-1} of carbohydrate. Test carbohydrates were sterilized separately and added after cooling. To minimize substrate changes, acid-swollen cellulose, ball-milled cellulose and xylan were not autoclaved. The antibiotic chloramphenicol (200 mg L^{-1}) was added to prevent the growth of contaminating organisms. Flasks were inoculated (10% v/v) with 24-h broth cultures (50 g L^{-1} glucose) and incubated at 35° C with agitation (100 rpm) for 24-96 h. To monitor cultures, samples were removed daily to determine the ethanol concentrations by gas chromatography (Dombek *et al.* (1986) *Appl. Environ. Microbiol.* 52:975-981).

Methods for Isolating and Identifying a Surrogate Promoter

In order to identify random fragments of *Z. mobilis* that would serve as surrogate promoters for the expression of heterologous genes in *Klebsiella* and other host cells, a vector for the efficient cloning of candidate promoters was constructed as described in Example 1 (see also, Ingram *et al.* (1988) *Appl. Environ. Microbiol.* 54:397-404).

Next, *Sau*3AI digested *Z. mobilis* DNA fragments were ligated into the *Bam*HI site of pLOI2171 to generate a library of potential promoters. These plasmids were transformed into *E. coli* DH5α for initial screening. Of the 18,000 colonies individually tested on CMC plates, 75 clones produced larger yellow zones than the control (pLOI2173). Plasmids from these 75 clones were then transformed into *K. oxytoca* M5A1, retested, and found to express high levels of *celZ* in this second host.

Recombinant Klebsiella Hosts for Producing Polysaccharase

The high expressing clones (pLOI2177 to pLOI2194) with the largest zones on CMC plates indicating *celZ* expression were grown in LB broth and assayed for glucanase activity (Table 5).

TABLE 5. Evaluation of promoters for *celZ* expression and secretion in *K. oxytoca* M5A1

Plasmids[a]	No secretion genes		Secretion genes present (pCPP2006)	
	Total activity (IU L^{-1})[b]	Secreted activity (IU L^{-1})	Total activity (IU L^{-1})	Secreted activity (IU L^{-1})
pLOI2173	2,450	465	3,190	1,530
pLOI2177	19,700	3,150	32,500	13,300
pLOI2178	15,500	2,320	21,300	11,500
pLOI2179	15,400	2,310	21,400	12,000
pLOI2180	21,400	3,210	30,800	13,600
pLOI2181	15,600	2,490	21,000	11,800
pLOI2182	19,600	3,130	31,100	14,000

pLOI2183	20,700	3,320	32,000	14,000
pLOI2184	15,500	2,480	21,200	11,900
pLOI2185	15,100	2.420	24.600	11,500
pLOI2186	17,000	2.380	25,700	13,400
pLOI2187	15,800	2,210	24,500	12,200
pLOI2188	18,200	2,180	25,600	12,000
pLOI2189	14,800	2.360	27,100	12,700
pLOI2190	16,100	2.410	26,500	12,500
pLOI2191	15,800	2.210	25,000	12,400
pLOI2192	15,100	1,810	24,900	12,500
pLOI2193	16.700	2.010	24,600	12.800
pLOI2194	15,400	2.770	21,500	11.900

[a] pLOI2173 contains the *celZ* gene with the original promoter, all others contain the *celZ* gene with a *Z. mobilis* DNA fragment which serves as a surrogate promoter.

[b] Glucanase (CMCase) activities were determined after 16 h of growth at 30°C.

Activities with these plasmids were up to 8-fold higher than with the control plasmid containing a native *celZ* promoter (pLOI2173). The four plasmids which produced the largest zones (pLOI2177, pLOI2180, pLOI2182 and pLOI2183) also produced the highest total glucanase activities (approximately 20,000 IU L^{-1}) released into the broth. One of these plasmids, pLOI2183, was selected for chromosomal integration.

Chromosomal Integration of a Polysaccharase Gene

To stably incorporate a desirable polysaccharase gene into a suitable host cell, *e.g.*, *Klebsiella* P2 strain, a novel vector (pLOI2306) was constructed to facilitate the isolation of a DNA fragment which lacked all replication functions but contained the *celZ* gene with surrogate promoter, a selectable marker, and a homologous DNA fragment for integration (Figure 7). Two *Asc*I sites were added to pUC19 by inserting a linker (GGCGCGCC; SEQ ID NO: 11) into Klenow-treated *Nde*I and *Sap*I sites which flank the polylinker region to produce pLOI2302. A blunt fragment containing the *tet*

resistance marker gene from pBR322 (excised with *Eco*RI and *Ava*I, followed by Klenow treatment) was cloned into the *Pst*I site of pLOI2302 (cut with *Pst*I, followed by Klenow treatment) to produce pLOI2303. To this plasmid was ligated a blunt fragment of *K. oxytoca* M5A1 chromosomal DNA (cut with *Eco*RI and made blunt with Klenow treatment) into the *Sma*I site of pLOI2303 to produce (pLOI2305). The *Eco*RI - *Sph*I fragment (Klenow treated) containing the surrogate *Z. mobilis* promoter and *celZ* gene from pLOI2183 was ligated into the *Eco*RI site of pLOI2305 *(Eco*RI, Klenow treatment) to produce pLOI2306. Digestion of pLOI2306 with *Asc*I produced two fragments, the larger of which contained the *celZ* gene with a surrogate promoter, *tet* gene, and chromosomal DNA fragment for homologous recombination. This larger fragment (10 kbp) was purified by agarose gel electrophoresis, circularized by self-ligation, and electroporated into the *Klebsiella* strain P2 and subsequently grown under selection for tetracycline resistance. The resulting 21 tetracycline-resistant colonies were purified and tested on CMC plates for glucanase activity. All were positive with large zones indicating functional expression of the *celZ* gene product.

Clones used to produce the recombinant strains were tested for the presence of unwanted plasmids by transforming DH5α with plasmid DNA preparations and by gel electrophoresis. No transformants were obtained with 12 clones tested. However, two of these strains were subsequently found to contain large plasmid bands which may contain *celZ* and these were discarded. Both strains with large plasmids contained DNA which could be sequenced with T7 and M13 primers confirming the presence of multicopy plasmids. The remaining ten strains contain integrated *celZ* genes and could not be sequenced with either primer.

The structural features of the novel vector pLOI2306 are schematically shown in Fig. 8 and the nucleotide sequence of the vector, including various coding regions (*i.e.*, of the genes *celZ*, *bla*, and *tet*), are indicated in SEQ ID NO: 12 of the sequence listing. Nucleotide base pairs 3282-4281, which represent non-coding sequence downstream of the *celZ* gene (obtained from *E. chrysanthemi*), and base pairs 9476-11544 which represent a portion of the non-coding target sequence obtained from *K. oxytoca* M5A1, remain to be sequenced using standard techniques (*e.g.*, as described in Sambrook, J. *et al.*, T. *Molecular Cloning: A Laboratory Manual. 2nd, ed., Cold Spring Harbor Laboratory,* Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, (1989);

Current Protocols in Molecular Biology, eds. Ausubel *et al.*, John Wiley & Sons (1992)). For example, sufficient flanking sequence on either side of the aforementioned unsequenced regions of the pLOI2306 plasmid is provided such that sequencing primers that correspond to these known sequences can be synthesized and used to carry out standard sequencing reactions using the pLOI2306 plasmid as a template.

Alternatively, it will be understood by the skilled artisan that these unsequenced regions can also be determined even in the absence of the pLOI2306 plasmid for use as a template. For example, the remaining *celZ* sequence can be determined by using the sequence provided herein (e.g., nucleotides 1452-2735 of SEQ ID NO: 12) for synthesizing probes and primers for, respectively, isolating a *celZ* containing clone from a library comprising *E. chrysanthemi* sequences and sequencing the isolated clone using a standard DNA sequencing reaction. Similarly, the remaining target sequence can be determined by using the sequence provided herein (*e.g.*, nucleotides 8426-9475 of SEQ ID NO: 12) for synthesizing probes and primers for, respectively, isolating a clone containing target sequence from a library comprising *K. oxytoca* M5A1 *Eco*RI fragments (*e.g.*, of the appropriate size) and sequencing the isolated clone using a standard DNA sequencing reaction (a source of *K. oxytoca* M5A1 would be, *e.g.*, ATCC 68564 cured free of any plasmid using standard techniques). The skilled artisan will further recognize that the making of libraries representative of the cDNA or genomic sequences of a bacterium and the isolation of a desired nucleic acid fragment from such a library (*e.g.*, a cDNA or genomic library), are well known in the art and are typically carried out using, *e.g.*, hybridization techniques or the polymerase chain reaction (PCR) and all of these techniques are standard in the art (see, *e.g.*, Sambrook, J. *et al.*, T. *Molecular Cloning: A Laboratory Manual. 2nd, ed., Cold Spring Harbor Laboratory,* Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, (1989); *Current Protocols in Molecular Biology*, eds. Ausubel *et al.*, John Wiley & Sons (1992); *Oligonucleotide Synthesis* (M.J. Gait, Ed. 1984); and *PCR Handbook Current Protocols in Nucleic Acid Chemistry*, Beaucage, Ed. John Wiley & Sons (1999) (Editor)).

Heterologous Gene Expression Using a Surrogate Promoter and Integrated or Plasmid-Based Constructs

The ten integrated strains (SZ1-SZ10) were investigated for glucanase production in LB sorbitol broth (Table 6). All produced 5,000-7,000 IUL^{-1} of active enzyme. Although this represents twice the activity expressed from plasmid pLOI2173 containing the native *celZ* promoter, the integrated strains produced only 1/3 the glucanase activity achieved by P2 (pLOI2183) containing the same surrogate *Z. mobilis* promoter (Table 5). The reduction in glucanase expression upon integration may be attributed to a decrease in copy number (*i.e.*, multiple copy plasmid versus a single integrated copy).

Secretion of Glucanase EGZ

K. oxytoca contains a native Type II secretion system for pullulanase secretion (Pugsley (1993) *Microbiol. Rev.* 57:50-108), analogous to the secretion system encoded by the *out* genes in *Erwinia chrysanthemi* which secrete pectate lyases and glucanase (EGZ) (Barras *et al.* (1994) *Annu. Rev. Phytopathol.* 32:201-234; He *et al.* (1991) *Proc. Natl. Acad. Sci. USA.* 88: 1079- 1083). Type II secretion systems are typically very specific and function poorly with heterologous proteins (He *et al.* (1991) *Proc. Natl. Acad. Sci. USA.* 88: 1079- 1083; Py *et al.* (1991) *FEMS Microbiol. Lett.* 79:315-322; Sauvonnet *et al.* (1996) *Mol. Microbiol.* 22: 1-7). Thus as expected, recombinant *celZ* was expressed primarily as a cell associated product with either M5A1 (Table 5) or P2 (Table 6) as the host. About 1/4 (12-26%) of the total recombinant EGZ activity was recovered in the broth. With *E. coli* DH5α, about 8-12% of the total extracellular EGZ was present. Thus the native secretion system in *K. oxytoca* may facilitate partial secretion of recombinant EGZ.

To further improve secretion of the desired products, type II secretion genes *(out* genes) from E. *chrysanthemi* EC16 were introduced (*e.g.*, using pCPP2006) to facilitate secretion of the recombinant EGZ from strain P86021 in ethanologenic strains of *K. oxytoca* (Table 5 and Table 6). For most strains containing plasmids with *celZ,* addition of the *out* genes resulted in a 5-fold increase in extracellular EGZ and a 2-fold increase in total glucanase activity. For strains with integrated *celZ,* addition of the *out* genes resulted in a 10-fold increase in extracellular EGZ and a 4-fold increase in total

glucanase activity. In both cases, the *out* genes facilitated secretion of approximately half the total glucanase activity. The increase in EGZ activity resulting from addition of the *out* genes may reflect improved folding of the secreted product in both plasmid and integrated *celZ* constructs. The smaller increase observed with the pUC-based derivatives may result from plasmid burden and competition for export machinery during the production of periplasmic β-lactamase from the *bla* gene on this high copy plasmid.

Two criteria were used to identify the best integrated strains of P2, growth on solid medium containing high levels of chloramphenicol (a marker for high level expression of the upstream *pdc* and *adhB* genes) and effective secretion of glucanase with the *out* genes. Two recombinant strains were selected for further study, SZ2 and SZ6. Both produced 24,000 IU $^{L-1}$ of glucanase activity, equivalent to approximately 5% of the total cellular protein (Py *et al.* (1991) *Protein Engin.* 4:325-333).

Substrate Depolymerization

The substrate depolymerization of the recombinant EGZ was determined to be excellent when applied to a CMC source (Table 7). When applied to acid swollen cellulose, the activity of the glucanase was less than 10% of the activity measured for CMC activity. Little activity was noted when the polysaccharase was applied to Avicel or xylan. However, when allowed to digest overnight, the EGZ polysaccharase resulted in a measurable reduction in average polymer length for all substrates. CMC and acid-swollen cellulose were depolymerized to an average length of 7 sugar residues. These cellulose polymers of 7 residues are marginally soluble and, ideally, may be further digested for efficient metabolization (Wood *et al.* (1992) *Appl. Environ. Microbiol.* 58:2103-2110). The average chain length of ball-milled cellulose and Avicel was reduced to 1/3 of the original length while less than a single cut was observed per xylan polymer.

TABLE 6. Comparison of culture growth, glucanase production, and secretion from ethanologenic *K. oxytoca* strains containing integrated *celZ*

Strains	Growth on solid medium (600 mg L^{-1} CM)	Glucanase production and secretion (IU L^{-1})			
		No secretion system		Adding secretion system (pCPP2006)	
		Total activity	Secreted activity	Total activity	Secreted activity
P2	++++	0	0	0	0
SZ1	++	6,140	1,600	26,100	14,300
SZ2	++++	6,460	1,160	23,700	11,400
SZ3	+++	5,260	1,320	18,400	8,440
SZ4	+++	7,120	1,070	23,200	9,990
SZ5	+	6,000	1,080	29,300	15,500
SZ6	++++	7,620	1,520	24,300	11,900
SZ7	+	6,650	1,330	28,800	15,500
SZ8	+++	7,120	854	28,700	14,900
SZ9	++	7,530	1,130	26,700	12,800
SZ10	+++	4,940	939	17,000	6,600

Glucanase (CMCase) activities were determined after 16 h of growth at 30°C.

TABLE 7. Depolymerization of vari us substrates by EGZ from cell free broth of strain SZ6 (pCPP2006)

Substrates	Enzyme activity (IU/L)	Estimated degree of polymerization	
		Before digestion	After digestion
Carboxymethyl cellulose	13,175	224	7
Acid-Swollen cellulose	893	87	7
Ball-milled cellulose	200	97	28
Avicel	41	104	35
Xylan from oat spelts	157	110	78

Strain SZ6 (pCPP2006) was grown in LB-sorbitol broth for 16 h as a source of secreted EGZ.

Fermentation

To be useful, addition of *celZ* and *out* genes to strain P2 must not reduce the fermentative ability of the resulting biocatalyst. A comparison was made using glucose and cellobiose (Table 8). All strains were equivalent in their ability to ferment these sugars indicating a lack of detrimental effects from the integration of *celZ* or addition of pCPP2006. These strains were also examined for their ability to convert acid-swollen cellulose directly into ethanol. The most active construct SZ6 (pCPP2006) produced a small amount of ethanol (3.9 g L^{-1}) from amorphous cellulose. Approximately 1.5 g L^{-1} ethanol was present initially at the time of inoculation for all strains. This decreased with time to zero for all strains except SZ6 (pCPP2006). Thus the production of 3.9 g L^{-1} ethanol observed with SZ6 (pCPP2006) may represent an underestimate of total ethanol production. However, at best, this represents conversion of only a fraction of the polymer present. It is likely that low levels of glucose, cellobiose, and cellotriose were produced by EGZ hydrolysis of acid swollen cellulose and fermented. These compounds can be metabolized by the native phosphoenolpyruvate-dependent phosphotransferase system in *K. oxytoca* (Ohta K *et al.*, (1991) *Appl. Environ. Microbiol.* 57:893-900; Wood *et al.* (1992) *Appl. Environ. Microbiol.* 58:2103-2110).

TABLE 8. Ethanol production by strain SZ6 containing *out* genes (pCPP2006) and integrated *celZ* using various substrates (50 g L^{-1})

Strains	Ethanol production (g L^{-1})		
	Glucose	Cellobiose	Acid-swollen cellulose
P2	22.9	22.7	0
P2 (pCPP2006)	22.6	21.3	0
SZ6	21.5	19.7	0
SZ6 (pCPP2006)	22.7	21.2	3.9

Initial ethanol concentrations at the time of inoculation were approximately 1.5 g L^{-1} for all cultures. With acid swollen cellulose as a substrate, these levels declined to 0 after 72 h of incubation for all strains except SZ6 (pCP206).

Equivalents

Those skilled in the art will recognize, or be able to ascertain using no more than routine experimentation, many equivalents to the specific embodiments of the invention described herein. Such equivalents are intended to be encompassed by the following claims. Moreover, any number of genetic constructs, host cells, and methods described in United States Patent Nos. 5,821,093; 5,482,846; 5,424,202; 5,028,539; 5,000,000; 5,487,989, 5,554,520, and 5,162,516, may be employed in carrying out the present invention and are hereby incorporated by reference.

What is claimed is:

1. A recombinant host cell comprising:

a first heterologous polynucleotide segment comprising a sequence encoding a polysaccharase polypeptide under the transcriptional control of a surrogate promoter, said promoter capable of causing increased expression of said polysaccharase polypeptide; and

a second heterologous polynucleotide segment comprising a sequence encoding a secretory polypeptide,

wherein expression of said first and second polynucleotide segments results in the increased production of a polysaccharase by the recombinant host cell.

2. The recombinant host cell of claim 1 wherein production is selected from the group consisting of activity, amount, and a combination thereof.

3. The recombinant host cell of claim 2 wherein said polysaccharase polypeptide is secreted.

4. The recombinant host cell of claim 2 wherein said host cell is a bacterial cell.

5. The recombinant host cell of claim 4 wherein said host cell is a Gram-negative bacterial cell.

6. The recombinant host cell of claim 5 wherein said host cell is a facultatively anaerobic bacterial cell.

7. The recombinant host cell of claim 6 wherein said host cell is selected from the family Enterobacteriaceae.

8. The recombinant host cell of claim 7 wherein said host is selected from the group consisting of *Escherichia* and *Klebsiella*.

9. The recombinant host cell of claim 8 wherein said *Escherichia* is selected from the group consisting of *E. coli* B, *E. coli* DH5α, *E. coli* KO4 (ATCC 55123), *E. coli* KO11 (ATCC 55124), *E. coli* KO12 (ATCC 55125) and *E. coli* LY01, *K. oxytoca* M5A1, and *K. oxytoca* P2 (ATCC 55307).

10. The recombinant host cell of claim 2 wherein said polysaccharase is selected from the group consisting of glucanase, endoglucanase, exoglucanase, cellobiohydrolase, β-glucosidase, endo-1,4-β-xylanase, α-xylosidase, α-glucuronidase, α-L-arabinofuranosidase, acetylesterase, acetylxylanesterase, α-amylase, β-amylase, glucoamylase, pullulanase, β-glucanase, hemicellulase, arabinosidase, mannanase, pectin hydrolase, pectate lyase, or a combination thereof.

11. The recombinant host cell of claim 10 wherein said polysaccharase is glucanase.

12. The recombinant host cell according to claim 10, wherein said polysaccharase is an expression product of a *celZ* gene.

13. The recombinant host cell of claim 12 wherein said *celZ* gene is derived from *Erwinia chrysanthemi*.

14. The recombinant host cell of claim 2 wherein said second heterologous polynucleotide segment comprises at least one *pul* gene or *out* gene.

15. The recombinant host cell of claim 2 wherein said second heterologous polynucleotide segment is derived from a bacterial cell selected from the family Enterobacteriaceae.

16. The recombinant host cell of claim 15 wherein said bacterial cell is selected from the group consisting of *K. oxytoca*, *E. carotovora*, *E. carotovora* subspecies *carotovora*, *E. carotovora* subspecies *atroseptica*, and *E. chrysanthemi*.

17. The recombinant host cell of claim 2 wherein said surrogate promoter comprises a polynucleotide fragment derived from *Zymomonas mobilis*.

18. The recombinant host cell of claim 17 wherein said surrogate promoter comprises a nucleic acid having the sequence provided in SEQ ID NO: 1, or a fragment thereof.

19. The recombinant host cell of any one of claims 1-18 wherein said host cell is ethanologenic.

20. A recombinant ethanologenic host cell comprising a heterologous polynucleotide segment encoding a polysaccharase under the transcriptional control of an exogenous surrogate promoter.

21. The recombinant host cell of claim 20 wherein said host cell is a bacterial cell.

22. The recombinant host cell of claim 21 wherein said host cell is a Gram-negative bacterial cell.

23. The recombinant host cell of claim 22 wherein said host cell is a facultatively anaerobic bacterial cell.

24. The recombinant host cell of claim 23 wherein said host cell is selected from the family Enterobacteriaceae.

25. The recombinant host cell of claim 24 wherein said host is selected from the group consisting of *Escherichia* and *Klebsiella*.

26. The recombinant host cell of claim 25 wherein said *Escherichia* and *Klebsiella* are selected from the group consisting of *E. coli* B, *E. coli* DH5α, *E. coli* KO4 (ATCC 55123), *E. coli* KO11 (ATCC 55124), *E. coli* KO12 (ATCC 55125), *E. coli* LY01, *K. oxytoca* M5A1 and *K. oxytoca* P2 (ATCC 55307).

27. The recombinant host cell of claim 20 wherein said polysaccharase is selected from the group consisting of glucanase, endoglucanase, exoglucanase, cellobiohydrolase, α-glucosidase, endo-1,4-α-xylanase, β-xylosidase, β-glucuronidase, α-L-arabinofuranosidase, acetylesterase, acetylxylanesterase, α-amylase, β-amylase, glucoamylase, pullulanase, β-glucanase, hemicellulase, arabinosidase, mannanase, pectin hydrolase, pectate lyase, or a combination thereof.

28. The recombinant host cell of claim 27 wherein said polysaccharase is glucanase.

29. The recombinant host cell according to claim 28 wherein said polysaccharase is an expression product of a *celZ* gene.

30. The recombinant host cell of claim 29 wherein said *celZ* gene is derived from *Erwinia chrysanthemi*.

31. The recombinant host cell of claim 20 wherein said surrogate promoter comprises a polynucleotide fragment derived from *Zymomonas mobilis*.

32. The recombinant host cell of claim 31 wherein said surrogate promoter comprises a polynucleotide segment having the sequence provided in SEQ ID NO: 1, or a fragment thereof.

33. A recombinant ethanologenic Gram-negative bacterial host cell comprising:

a first heterologous polynucleotide segment comprising a sequence encoding a first polypeptide; and

a second heterologous polynucleotide segment comprising a sequence encoding a secretory polypeptide,

wherein production of the first polypeptide by the host cell is increased.

34. The recombinant host cell of claim 33 wherein said first polypeptide is secreted.

35. The recombinant host cell of claim 33 wherein said host cell is a facultatively anaerobic bacterial cell.

36. The recombinant host cell of claim 35 wherein said host cell is selected from the family Enterobacteriaceae.

37. The recombinant bacterial host cell of claim 36 wherein said host cell is selected from the group consisting of *Escherichia* and *Klebsiella*.

38. The recombinant bacterial host cell of claim 37 wherein said *Escherichia* and *Klebsiella* are selected from the group consisting of *E. coli* B, *E. coli* DH5α, *E. coli* KO4 (ATCC 55123), *E. coli* KO11 (ATCC 55124), *E. coli* KO12 (ATCC 55125) *E. coli* LY01, *K. oxytoca* M5A1, and *K. oxytoca* P2 (ATCC 55307).

39. The recombinant bacterial host cell of claim 33 wherein said first polypeptide is a polysaccharase.

40. The recombinant bacterial host cell of claim 39 wherein said polysaccharase is of increased activity.

41. The recombinant host cell of claim 39 wherein said polysaccharase is selected from the group consisting of glucanase, endoglucanase, exoglucanase, cellobiohydrolase, α-glucosidase, endo-1,4-α-xylanase, β-xylosidase, β-glucuronidase, α-L-arabinofuranosidase, acetylesterase, acetylxylanesterase, α-amylase, β-amylase, glucoamylase, pullulanase, β-glucanase, hemicellulase, arabinosidase, mannanase, pectin hydrolase, pectate lyase, or a combination thereof.

42. The recombinant host cell of claim 41 wherein said polysaccharase is glucanase.

43. The recombinant host cell according to claim 42 wherein said glucanase is an expression product of a *celZ* gene.

44. The recombinant host cell of claim 43 wherein said *celZ* gene is derived from *Erwinia chrysanthemi*.

45. The recombinant host cell of claim 33 wherein said second heterologous polynucleotide segment comprises at least one *pul* gene or *out* gene.

46. The recombinant host cell of claim 45 wherein said second heterologous polynucleotide segment is derived from a bacterial cell selected from the family Enterobacteriaceae.

47. The recombinant host cell of claim 46 wherein said bacterial cell is selected from the group consisting of *K. oxytoca*, *E. carotovora*, *E. carotovora* subspecies *carotovora*, *E. carotovora* subspecies *atroseptica*, and *E. chrysanthemi*.

48. A method for enzymatically degrading an oligosaccharide comprising the steps of:

providing a oligosaccharide; and

contacting said oligosaccharide with a host cell comprising a first heterologous polynucleotide segment comprising a sequence encoding a polysaccharase under the transcriptional control of a surrogate promoter, said promoter capable of causing increased expression of said polysaccharase; and

a second heterologous polynucleotide segment comprising a sequence encoding a secretory polypeptide,

wherein expression of said first and second polynucleotide segments results in the increased production of polysaccharase activity by the recombinant host cell such that the oligosaccharide is enzymatically degraded.

49. The method of claim 48 wherein said polysaccharase is secreted.

50. The method of claim 48 wherein said host cell is ethanologenic.

51. The method of claim 48 wherein said method is conducted in an aqueous solution.

52. The method of claim 48 wherein said method is used for simultaneous saccharification and fermentation.

53. The method of claim 48 wherein said oligosaccharide is selected from the group consisting of lignocellulose, hemicellulose, cellulose, pectin, and any combination thereof.

54. A method of identifying a surrogate promoter capable of increasing the expression of a gene-of-interest in a host cell, said method comprising:

fragmenting a genomic polynucleotide from an organism into one or more fragments;

placing said gene-of-interest under the transcriptional control of at least one fragment;

introducing said fragment and gene-of-interest into a host cell; and

identifying a host cell having increased expression of said gene-of-interest whereby said increased expression indicates that the fragment is a surrogate promoter.

55. A method of making a recombinant host cell for use in simultaneous saccharification and fermentation comprising:

introducing into said host cell a first heterologous polynucleotide segment comprising a sequence encoding a polysaccharase under the transcriptional control of a surrogate promoter, said promoter capable of causing increased expression of said polysaccharase; and

introducing into said host cell a second heterologous polynucleotide segment comprising a sequence encoding a secretory polypeptide,

wherein expression of said first and second polynucleotide segments results in the increased production of a polysaccharase by the recombinant host cell.

56. The recombinant host cell of claim 55 wherein production is selected from the group consisting of activity, amount, and a combination thereof.

57. The recombinant host cell of claim 55 or 56 wherein said polysaccharase polypeptide is secreted.

58. The method of claim 55, 56, or 57 wherein said host cell is ethanologenic.

59. A vector comprising the polynucleotide sequence of pLOI2306 (SEQ ID NO: 12).

60. A host cell having a vector comprising the polynucleotide sequence of pLOI2306 (SEQ ID NO: 12).

61. A method of making a recombinant host cell integrant comprising:

introducing into said host cell a vector comprising the polynucleotide sequence of pLOI2306 (SEQ ID NO: 12); and

identifying a host cell having said vector stably integrated.

62. A method for expressing a polysaccharase in a host cell comprising:

introducing into said host cell a vector comprising the polynucleotide sequence of pLOI2306 (SEQ ID NO: 12); and

identifying a host cell expressing said polysaccharase.

63. The method of any one of claims 60-62 wherein said host cell is ethanologenic.

64. A method for producing ethanol from an oligosaccharide source comprising,

contacting said oligosaccharide source with a ethanologenic host cell comprising a first heterologous polynucleotide segment comprising a sequence encoding a polysaccharase under the transcriptional control of a surrogate promoter, said promoter capable of causing increased expression of said polysaccharase; and

a second heterologous polynucleotide segment comprising a sequence encoding a secretory polypeptide,

wherein expression of said first and second polynucleotide segments results in the increased production of polysaccharase activity by the ethanologenic cell such that the oligosaccharide source is enzymatically degraded and fermented into ethanol.

65. The host cell of claim 64 wherein said polysaccharase is selected from the group consisting of glucanase, endoglucanase, exoglucanase, cellobiohydrolase, α-glucosidase, endo-1,4-α-xylanase, β-xylosidase, β-glucuronidase, α-L-arabinofuranosidase, acetylesterase, acetylxylanesterase, α-amylase, β-amylase, glucoamylase, pullulanase, β-glucanase, hemicellulase, arabinosidase, mannanase, pectin hydrolase, pectate lyase, or a combination thereof.

66. The host cell of claim 65 wherein said polysaccharase is glucanase.

67. The host cell according to claim 66 wherein said glucanase is an expression product of a *celZ* gene.

68. The host cell of claim 67 wherein said *celZ* gene is derived from *Erwinia chrysanthemi*.

69. The host cell of claim 64 wherein said second heterologous polynucleotide segment comprises at least one *pul* gene or *out* gene.

70. The host cell of claim 64 wherein said host cell is selected from the family Enterobacteriaceae.

71. The host cell of claim 64 wherein said host cell is selected from the group consisting of *Escherichia* and *Klebsiella*.

72. The host cell of claim 64, wherein said host cell is selected from the group consisting of *E. coli* KO4 (ATCC 55123), *E. coli* KO11 (ATCC 55124), *E. coli* KO12 (ATCC 55125), *K. oxytoca* M5A1, and *K. oxytoca* P2 (ATCC 55307).

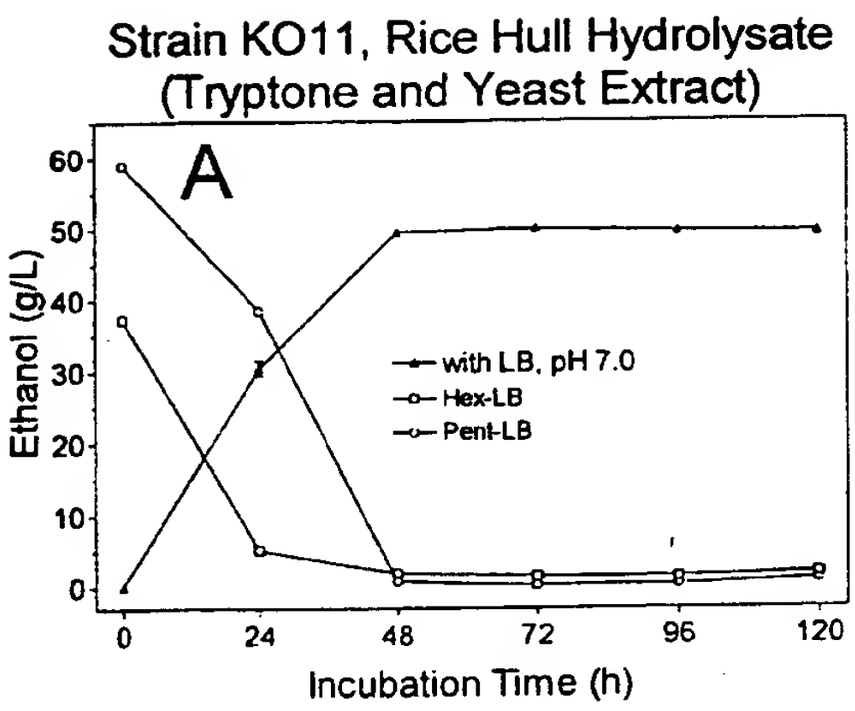
73. The host cell of claim 64, wherein said polysaccharase is of increased activity.

74. The method of claim 64, wherein said method is conducted in an aqueous solution.

75. The method of claim 64, wherein said oligosaccharide is selected from the group consisting of lignocellulose, hemicellulose, cellulose, pectin, and any combination thereof.

76. The method according to claim 64, wherein said first heterologous polynucleotide segment is, or derived from, pLOI2306 (SEQ ID NO: 12).

Fig. 1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Pent-LB
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72
96
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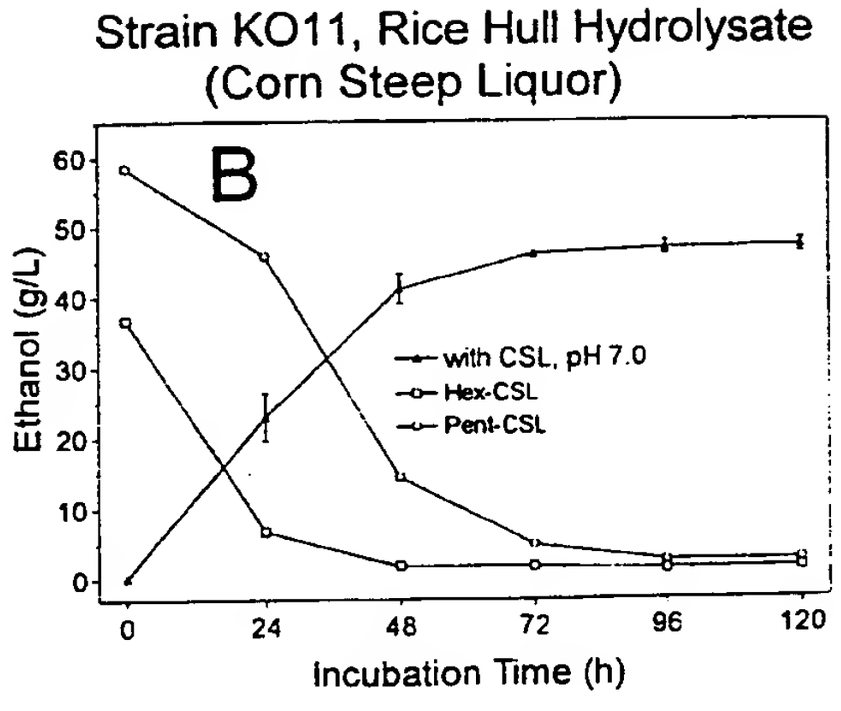
Incubation Time (h)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10
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48
72
96
120
Incubation Time (h)

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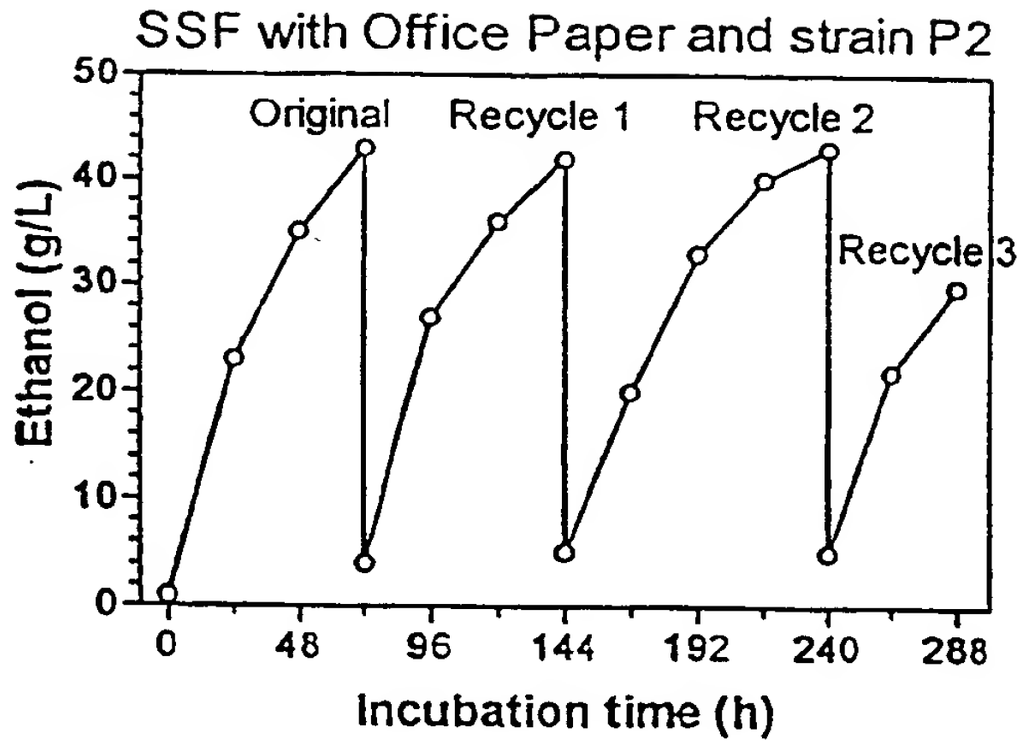
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Incubation time (h)

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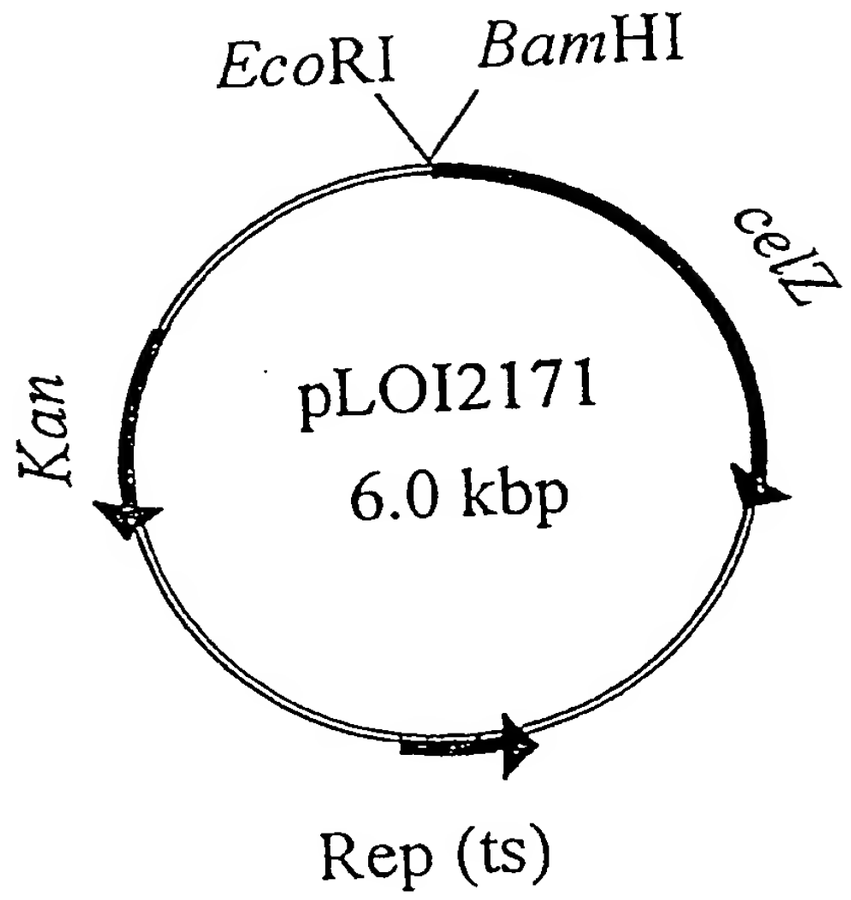

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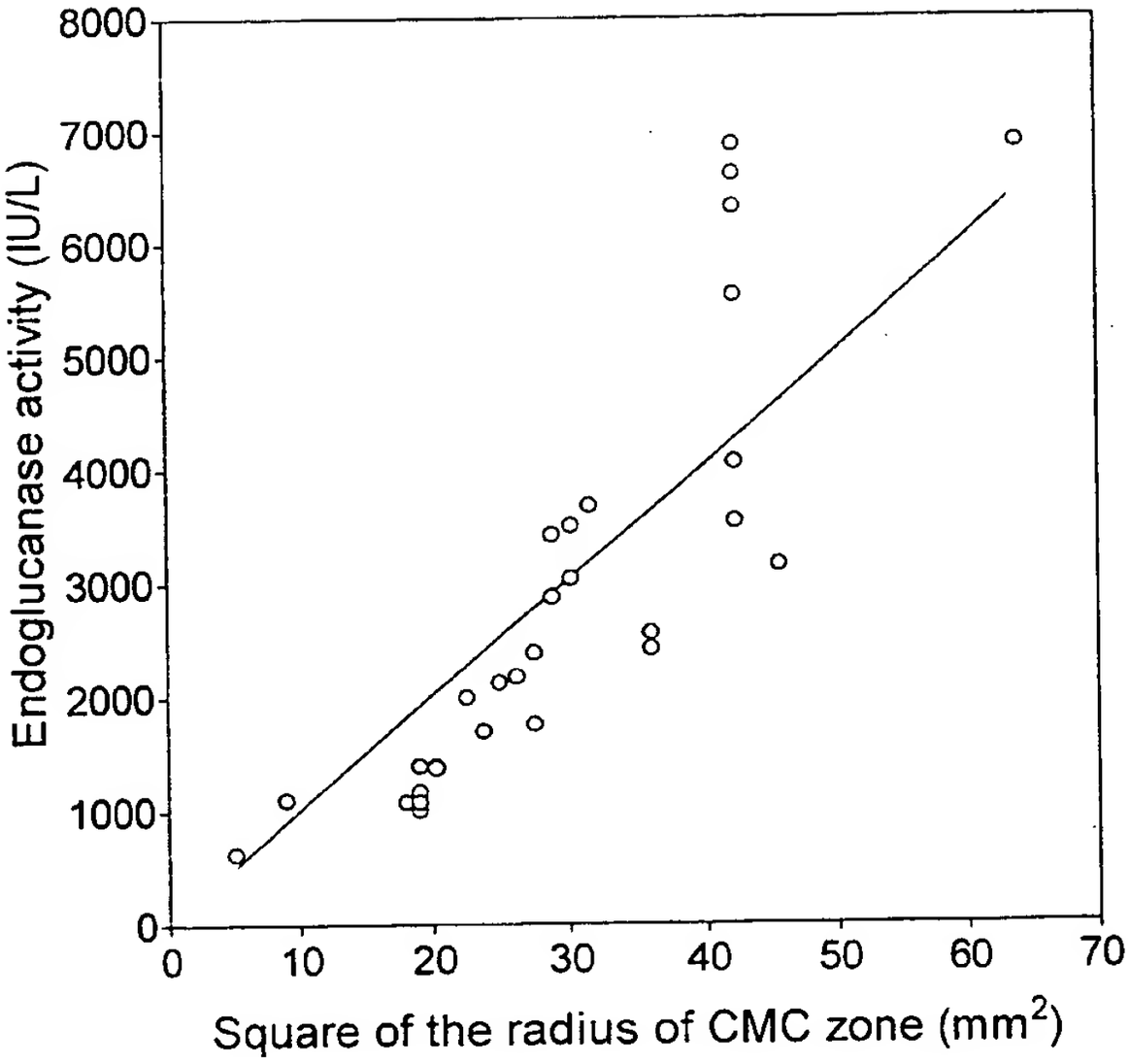
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Square of the radius of CMC zone (mm2)

Fig. 5

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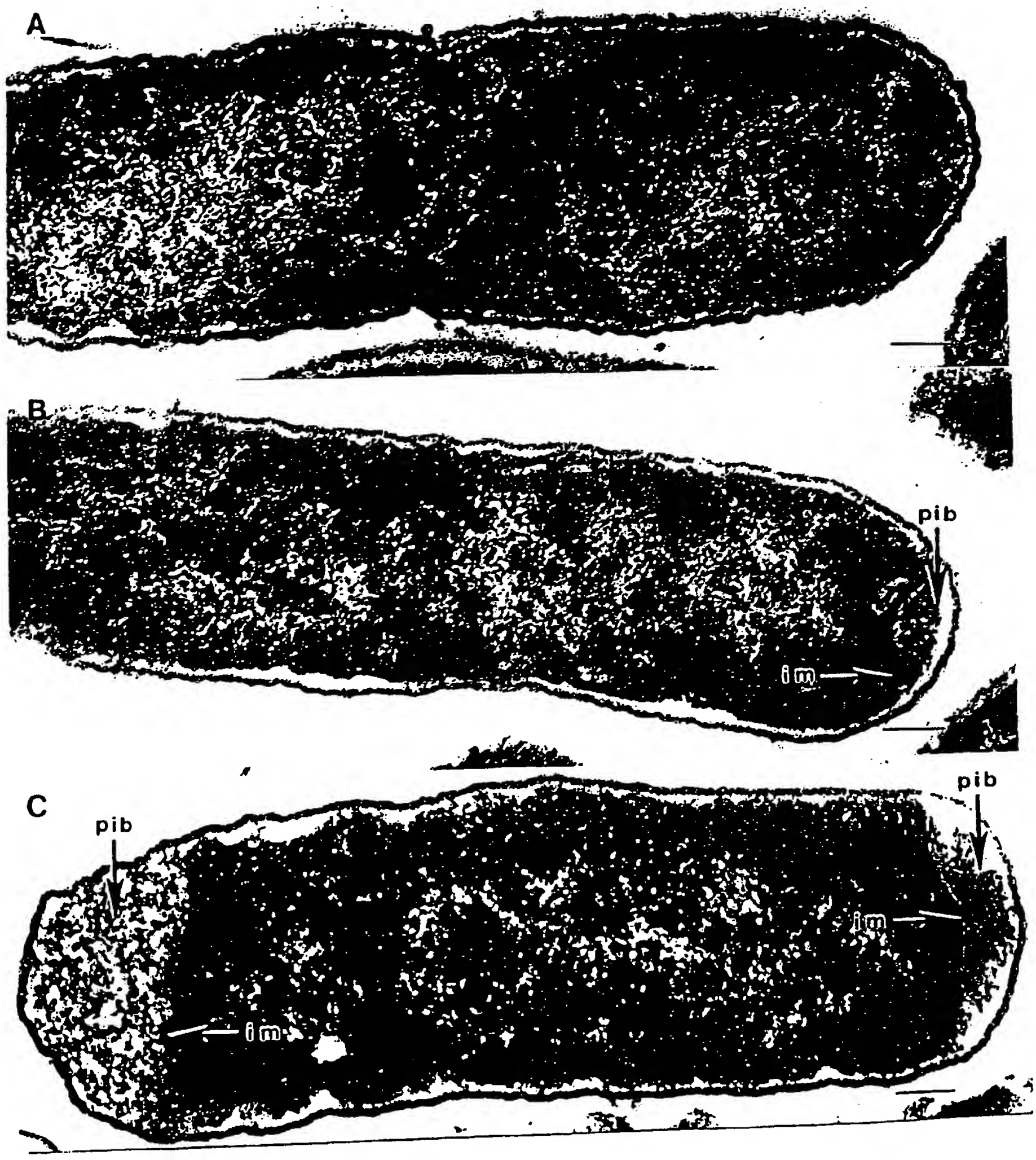

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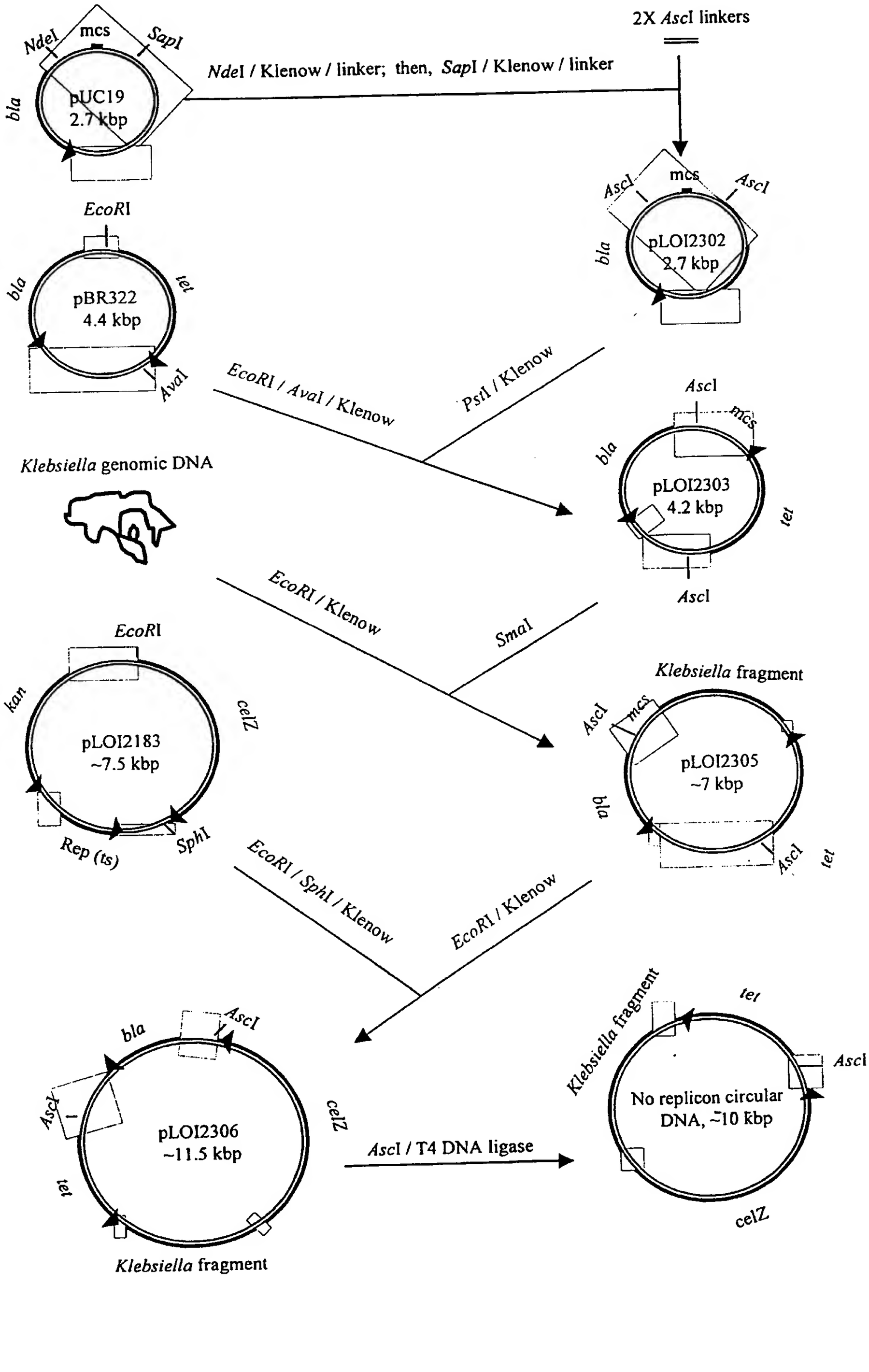
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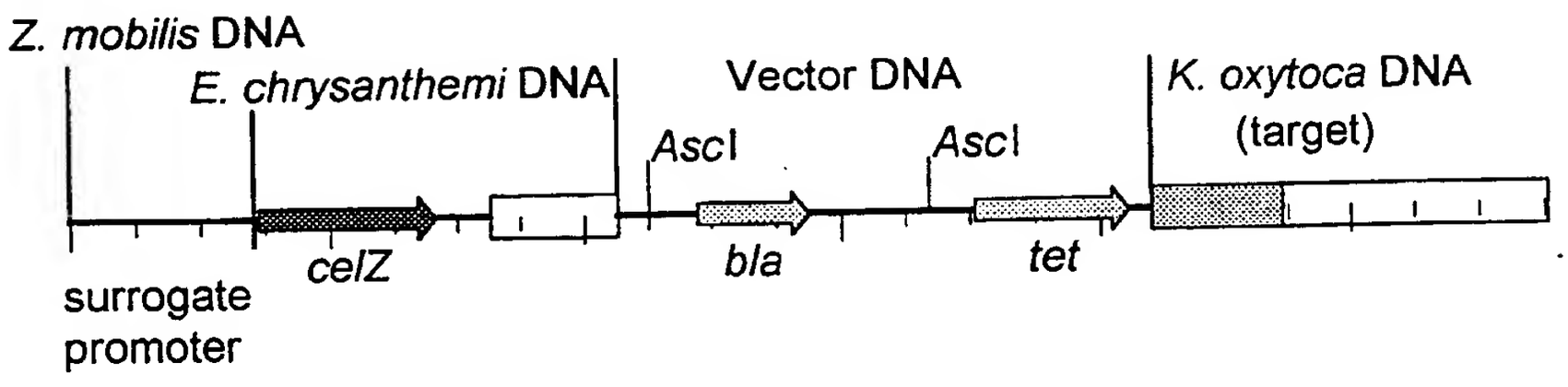
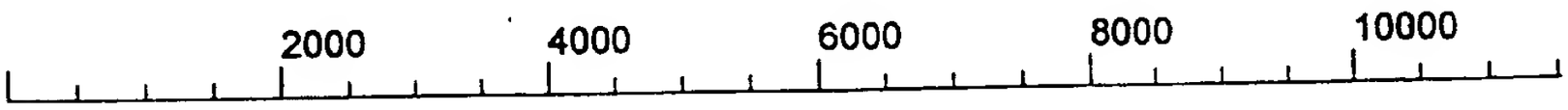
Fig. 8





pLOI2306 (11520 bps)

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     15                  20                  25

tta gga tta agc ctt gcc tgc ctt tcc agt aat gcc tgg gcg agt gtt   1586
Leu Gly Leu Ser Leu Ala Cys Leu Ser Ser Asn Ala Trp Ala Ser Val
 30                  35                  40                  45

gag ccg tta tcc gtt agc ggc aat aaa atc tac gca ggt gaa aaa gcc   1634
Glu Pro Leu Ser Val Ser Gly Asn Lys Ile Tyr Ala Gly Glu Lys Ala
                 50                  55                  60

aaa agt ttt gcc ggc aac agc tta ttc tgg agt aat aat ggt tgg ggt   1682
Lys Ser Phe Ala Gly Asn Ser Leu Phe Trp Ser Asn Asn Gly Trp Gly
             65                  70                  75

ggg gaa aaa ttc tac aca gcc gat acc gtt gcg tcg ctg aaa aaa gac   1730
Gly Glu Lys Phe Tyr Thr Ala Asp Thr Val Ala Ser Leu Lys Lys Asp
         80                  85                  90

tgg aaa tcc agc att gtt cgc gcc gct atg ggc gtt cag gaa agc ggt   1778
Trp Lys Ser Ser Ile Val Arg Ala Ala Met Gly Val Gln Glu Ser Gly
     95                  100                 105

ggt tat ctg cag gac ccg gct ggc aac aag gcc aaa gtt gaa aga gtg   1826
Gly Tyr Leu Gln Asp Pro Ala Gly Asn Lys Ala Lys Val Glu Arg Val
110                 115                 120                 125

gtg gat gcc gca atc gcc aac gat atg tat gtg att att gac tgg cac   1874
Val Asp Ala Ala Ile Ala Asn Asp Met Tyr Val Ile Ile Asp Trp His
                130                 135                 140

tca cat tct gca gaa aac aat cgc agt gaa gcc att cgc ttc ttc cag   1922
Ser His Ser Ala Glu Asn Asn Arg Ser Glu Ala Ile Arg Phe Phe Gln
            145                 150                 155

gaa atg gcg cgc aaa tat ggc aac aag ccg aat gtc att tat gaa atc   1970
Glu Met Ala Arg Lys Tyr Gly Asn Lys Pro Asn Val Ile Tyr Glu Ile
        160                 165                 170

tac aac gag ccg ctt cag gtt tca tgg agc aat acc att aaa cct tat   2018
Tyr Asn Glu Pro Leu Gln Val Ser Trp Ser Asn Thr Ile Lys Pro Tyr
    175                 180                 185

gcc gaa gcc gtg att tcc gcc att cgc gcc att gac ccg gat aac ctg   2066
Ala Glu Ala Val Ile Ser Ala Ile Arg Ala Ile Asp Pro Asp Asn Leu
190                 195                 200                 205
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att att gtc ggt acg ccc agt tgg tcg caa aac gtt gat gaa gcg tcg   2114
Ile Ile Val Gly Thr Pro Ser Trp Ser Gln Asn Val Asp Glu Ala Ser
                210                 215                 220

cgc gat cca atc aac gcc aag aat atc gcc tat acg ctg cat ttc tac   2162
Arg Asp Pro Ile Asn Ala Lys Asn Ile Ala Tyr Thr Leu His Phe Tyr
            225                 230                 235

gcg gga acc cat ggt gag tca tta cgc act aaa gcc cgc cag gcg tta   2210
Ala Gly Thr His Gly Glu Ser Leu Arg Thr Lys Ala Arg Gln Ala Leu
        240                 245                 250

aat aac ggt att gcg ctt ttc gtc acc gag tgg ggc gcc gtt aac gcg   2258
Asn Asn Gly Ile Ala Leu Phe Val Thr Glu Trp Gly Ala Val Asn Ala
    255                 260                 265

gac ggc aat ggc gga gtg aac cag aca gat acc gac gcc tgg gta acg   2306
Asp Gly Asn Gly Gly Val Asn Gln Thr Asp Thr Asp Ala Trp Val Thr
270                 275                 280                 285

ttc atg cgt gac aac aac atc agc aac gca aac tgg gcg tta aat gat   2354
Phe Met Arg Asp Asn Asn Ile Ser Asn Ala Asn Trp Ala Leu Asn Asp
                290                 295                 300

aaa agc gaa ggg gca tca acc tat tat ccg gac tct aaa aac ctg acc   2402
Lys Ser Glu Gly Ala Ser Thr Tyr Tyr Pro Asp Ser Lys Asn Leu Thr
            305                 310                 315

gag tcg ggt aaa ata gta aaa tcg atc att caa agc tgg cca tat aaa   2450
Glu Ser Gly Lys Ile Val Lys Ser Ile Ile Gln Ser Trp Pro Tyr Lys
        320                 325                 330

gcg ggc agc gcc gcc agt aca aca acc gat cag tca acc gat acc acc   2498
Ala Gly Ser Ala Ala Ser Thr Thr Thr Asp Gln Ser Thr Asp Thr Thr
    335                 340                 345

atg gca cca ccg ttg acg aac cga cca caa ccg aca cac cgg caa acc   2546
Met Ala Pro Pro Leu Thr Asn Arg Pro Gln Pro Thr His Arg Gln Thr
350                 355                 360                 365

gct gat tgc tgc aat gcc aac gtt tac ccc aac tgg gtt agc aaa gac   2594
Ala Asp Cys Cys Asn Ala Asn Val Tyr Pro Asn Trp Val Ser Lys Asp
                370                 375                 380

tgg gcg ggc cgg cag cga ctc ata acg aag cag gcc aat cga tcg tct   2642
Trp Ala Gly Arg Gln Arg Leu Ile Thr Lys Gln Ala Asn Arg Ser Ser
            385                 390                 395

aca aag gga acc tgt ata ccg caa act ggt aca ctt cat ccg ttc cgg   2690
Thr Lys Gly Thr Cys Ile Pro Gln Thr Gly Thr Leu His Pro Phe Arg
        400                 405                 410

gca gcg att cct cct ggg cac agg ttg gta gct gta act aat tga       2735
Ala Ala Ile Pro Pro Gly His Arg Leu Val Ala Val Thr Asn
    415                 420                 425

ttaatctttt caccccaaa ataacagggc tgcgattgca gcctgatacg caacattcca 2795
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ttacttaatt gcgttcaaaa gcgcccaaat ccggtgcgct gccttgtaac taatatgatt 2855

tctctttcgt acccgcgtta atcagctttg agttagccga cagacggaac agcgaggttg 2915

ccggcaacgt gccgtcatta tcacgagata cggtagccag cgaggtgtcc aggctgacga 2975

atcggacgcg gaagccgctg tccgtatcca tgagttgact cgcatccgca ttactgaccg 3035

ttgcagaagc agacagagac acgttgttgc ggaagtaatg tttctgtcct gactggacgt 3095

tgctcccgaa agcataatta atgccgtttt tatatgacgt gttatttatt accgtacgcc 3155

gccgcgttat tgttctggtc aaaacctttg ctcacgttgc caaacgcgac gcaacgggta 3215

atgcgatgat tgccgaccgc tggttcctcc cagtttgaac ccgttggcat tgccggcgaa 3275

cgcgctnnnn nnnnnnnnnn nnnnnnnnnn nnnnnnnnnn nnnnnnnnnn nnnnnnnnnn 3335

nnnnnnnnnn nnnnnnnnnn nnnnnnnnnn nnnnnnnnnn nnnnnnnnnn nnnnnnnnnn 3395

nnnnnnnnnn nnnnnnnnnn nnnnnnnnnn nnnnnnnnnn nnnnnnnnnn nnnnnnnnnn 3455

nnnnnnnnnn nnnnnnnnnn nnnnnnnnnn nnnnnnnnnn nnnnnnnnnn nnnnnnnnnn 3515

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nnnnnnnnnn nnnnnnnnnn nnnnnnnnnn nnnnnnnnnn nnnnnnnnnn nnnnnnnnnn 4175

nnnnnnnnnn nnnnnnnnnn nnnnnnnnnn nnnnnnnnnn nnnnnnnnnn nnnnnnnnnn 4235

nnnnnnnnnn nnnnnnnnnn nnnnnnnnnn nnnnnnnnnn nnnnnngatc ctctagagtc 4295

gacctgcagg aattcactgg ccgtcgtttt acaacgtcgt gactgggaaa accctggcgt 4355

tacccaactt aatcgccttg cagcacatcc ccctttcgcc agctggcgta atagcgaaga 4415

ggcccgcacc gatcgccctt cccaacagtt gcgcagcctg aatggcgaat ggcgcctgat 4475

gcggtatttt ctccttacgc atctgtgcgg tatttcacac cgcataggcg cgcctatggt 4535

gcactctcag tacaatctgc tctgatgccg catagttaag ccagccccga cacccgccaa 4595

```
cacccgctga cgcgccctga cgggcttgtc tgctcccggc atccgcttac agacaagctg 4655

tgaccgtctc cgggagctgc atgtgtcaga ggttttcacc gtcatcaccg aaacgcgcga 4715

gacgaaaggg cctcgtgata cgcctatttt tataggttaa tgtcatgata ataatggttt 4775

cttagacgtc aggtggcact tttcggggaa atgtgcgcgg aacccctatt tgtttatttt 4835

tctaaataca ttcaaatatg tatccgctca tgagacaata accctgataa atgcttcaat 4895

aatattgaaa aaggaagagt atg agt att caa cat ttc cgt gtc gcc ctt att 4948
                      Met Ser Ile Gln His Phe Arg Val Ala Leu Ile
                       1                                   10

ccc ttt ttt gcg gca ttt tgc ctt cct gtt ttt gct cac cca gaa acg    4996
Pro Phe Phe Ala Ala Phe Cys Leu Pro Val Phe Ala His Pro Glu Thr
                                20

ctg gtg aaa gta aaa gat gct gaa gat cag ttg ggt gca cga gtg ggt    5044
Leu Val Lys Val Lys Asp Ala Glu Asp Gln Leu Gly Ala Arg Val Gly
         30                                  40

tac atc gaa ctg gat ctc aac agc ggt aag atc ctt gag agt ttt cgc    5092
Tyr Ile Glu Leu Asp Leu Asn Ser Gly Lys Ile Leu Glu Ser Phe Arg
                       50

ccc gaa gaa cgt ttt cca atg atg agc act ttt aaa gtt ctg cta tgt    5140
Pro Glu Glu Arg Phe Pro Met Met Ser Thr Phe Lys Val Leu Leu Cys
 60                                      70

ggc gcg gta tta tcc cgt att gac gcc ggg caa gag caa ctc ggt cgc    5188
Gly Ala Val Leu Ser Arg Ile Asp Ala Gly Gln Glu Gln Leu Gly Arg
                  80                                  90

cgc ata cac tat tct cag aat gac ttg gtt gag tac tca cca gtc aca    5236
Arg Ile His Tyr Ser Gln Asn Asp Leu Val Glu Tyr Ser Pro Val Thr
                              100

gaa aag cat ctt acg gat ggc atg aca gta aga gaa tta tgc agt gct    5284
Glu Lys His Leu Thr Asp Gly Met Thr Val Arg Glu Leu Cys Ser Ala
         110                                 120

gcc ata acc atg agt gat aac act gcg gcc aac tta ctt ctg aca acg    5332
Ala Ile Thr Met Ser Asp Asn Thr Ala Ala Asn Leu Leu Leu Thr Thr
                       130

atc gga gga ccg aag gag cta acc gct ttt ttg cac aac atg ggg gat    5380
Ile Gly Gly Pro Lys Glu Leu Thr Ala Phe Leu His Asn Met Gly Asp
140                                     150

cat gta act cgc ctt gat cgt tgg gaa ccg gag ctg aat gaa gcc ata    5428
His Val Thr Arg Leu Asp Arg Trp Glu Pro Glu Leu Asn Glu Ala Ile
                  160                                 170

cca aac gac gag cgt gac acc acg atg cct gta gca atg gca aca acg    5476
Pro Asn Asp Glu Arg Asp Thr Thr Met Pro Val Ala Met Ala Thr Thr
                              180
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ttg cgc aaa cta tta act ggc gaa cta ctt act cta gct tcc cgg caa   5524
Leu Arg Lys Leu Leu Thr Gly Glu Leu Leu Thr Leu Ala Ser Arg Gln
        190                                     100

caa tta ata gac tgg atg gag gcg gat aaa gtt gca gga cca ctt ctg   5572
Gln Leu Ile Asp Trp Met Glu Ala Asp Lys Val Ala Gly Pro Leu Leu
                    110

cgc tcg gcc ctt ccg gct ggc tgg ttt att gct gat aaa tct gga gcc   5620
Arg Ser Ala Leu Pro Ala Gly Trp Phe Ile Ala Asp Lys Ser Gly Ala
120                                 130

ggt gag cgt ggg tct cgc ggt atc att gca gca ctg ggg cca gat ggt   5668
Gly Glu Arg Gly Ser Arg Gly Ile Ile Ala Ala Leu Gly Pro Asp Gly
                140                                 150

aag ccc tcc cgt atc gta gtt atc tac acg acg ggg agt cag gca act   5716
Lys Pro Ser Arg Ile Val Val Ile Tyr Thr Thr Gly Ser Gln Ala Thr
                            160

atg gat gaa cga aat aga cag atc gct gag ata ggt gcc tca ctg att   5764
Met Asp Glu Arg Asn Arg Gln Ile Ala Glu Ile Gly Ala Ser Leu Ile
        170                                     180

aag cat tgg taa ctgtcagacc aagtttactc atatatactt tagattgatt       5816
Lys His Trp
    185

taaaacttca tttttaattt aaaaggatct aggtgaagat ccttttttgat aatctcatga 5876

ccaaaatccc ttaacgtgag ttttcgttcc actgagcgtc agaccccgta gaaaagatca 5936

aaggatcttc ttgagatcct ttttttctgc gcgtaatctg ctgcttgcaa acaaaaaaac 5996

caccgctacc agcggtggtt tgtttgccgg atcaagagct accaactctt tttccgaagg 6056

taactggctt cagcagagcg cagataccaa atactgtcct tctagtgtag ccgtagttag 6116

gccaccactt caagaactct gtagcaccgc ctacatacct cgctctgcta atcctgttac 6176

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gcacgaggga gcttccaggg ggaaacgcct ggtatcttta tagtcctgtc gggtttcgcc 6476

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ataccgctcg ccgcagccga acgaccgagc gcagcgagtc agtgagcgag gaagcggcgc 6716

gccagcggaa gagcgcccaa tacgcaaacc gcctctcccc gcgcgttggc cgattcatta 6776
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atgcagctgg cacgacaggt ttcccgactg gaaagcgggc agtgagcgca acgcaattaa 6836

tgtgagttag ctcactcatt aggcacccca ggctttacac tttatgcttc cggctcgtat 6896

gttgtgtgga attgtgagcg gataacaatt tcacacagga aacagctatg accatgatta 6956

cgccaagctt gcatgccaat tctcatgttt gacagcttat catcgataag ctttaatgcg 7016

gtagtttatc acagttaaat tgctaacgca gtcaggcacc gtgt atg aaa tct aac  7072
                                                 Met Lys Ser Asn

aat gcg ctc atc gtc atc ctc ggc acc gtc acc ctg gat gct gta ggc   7120
Asn Ala Leu Ile Val Ile Leu Gly Thr Val Thr Leu Asp Ala Val Gly
                    10                                  20

ata ggc ttg gtt atg ccg gta ctg ccg ggc ctc ttg cgg gat atc gtc   7168
Ile Gly Leu Val Met Pro Val Leu Pro Gly Leu Leu Arg Asp Ile Val
                30

cat tcc gac agc atc gcc agt cac tat ggc gtg ctg cta gcg cta tat   7216
His Ser Asp Ser Ile Ala Ser His Tyr Gly Val Leu Leu Ala Leu Tyr
            40                                  50

gcg ttg atg caa ttt cta tgc gca ccc gtt ctc gga gca ctg tcc gac   7264
Ala Leu Met Gln Phe Leu Cys Ala Pro Val Leu Gly Ala Leu Ser Asp
                        60

cgc ttt ggc cgc cgc cca gtc ctg ctc gct tcg cta ctt gga gcc act   7312
Arg Phe Gly Arg Arg Pro Val Leu Leu Ala Ser Leu Leu Gly Ala Thr
    70                                  80

atc gac tac gcg atc atg gcg acc aca ccc gtc ctg tgg atc ctc tac   7360
Ile Asp Tyr Ala Ile Met Ala Thr Thr Pro Val Leu Trp Ile Leu Tyr
                90                                  100

gcc gga cgc atc gtg gcc ggc atc acc ggc gcc aca ggt gcg gtt gct   7408
Ala Gly Arg Ile Val Ala Gly Ile Thr Gly Ala Thr Gly Ala Val Ala
                        110

ggc gcc tat atc gcc gac atc acc gat ggg gaa gat cgg gct cgc cac   7456
Gly Ala Tyr Ile Ala Asp Ile Thr Asp Gly Glu Asp Arg Ala Arg His
            120                                 130

ttc ggg ctc atg agc gct tgt ttc ggc gtg ggt atg gtg gca ggc ccc   7504
Phe Gly Leu Met Ser Ala Cys Phe Gly Val Gly Met Val Ala Gly Pro
                        140

gtg gcc ggg gga ctg ttg ggc gcc atc tcc ttg cat gca cca ttc ctt   7552
Val Ala Gly Gly Leu Leu Gly Ala Ile Ser Leu His Ala Pro Phe Leu
    150                                 160

gcg gcg gcg gtg ctc aac ggc ctc aac cta cta ctg ggc tgc ttc cta   7600
Ala Ala Ala Val Leu Asn Gly Leu Asn Leu Leu Leu Gly Cys Phe Leu
                170                                 180

atg cag gag tcg cat aag gga gag cgt cga ccg atg ccc ttg aga gcc   7648
Met Gln Glu Ser His Lys Gly Glu Arg Arg Pro Met Pro Leu Arg Ala
                        190
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ttc aac cca gtc agc tcc ttc cgg tgg gcg cgg ggc atg act atc gtc   7696
Phe Asn Pro Val Ser Ser Phe Arg Trp Ala Arg Gly Met Thr Ile Val
           200                                 210

gcc gca ctt atg act gtc ttc ttt atc atg caa ctc gta gga cag gtg   7744
Ala Ala Leu Met Thr Val Phe Phe Ile Met Gln Leu Val Gly Gln Val
                       220

ccg gca gcg ctc tgg gtc att ttc ggc gag gac cgc ttt cgc tgg agc   7792
Pro Ala Ala Leu Trp Val Ile Phe Gly Glu Asp Arg Phe Arg Trp Ser
    230                             240

gcg acg atg atc ggc ctg tcg ctt gcg gta ttc gga atc ttg cac gcc   7840
Ala Thr Met Ile Gly Leu Ser Leu Ala Val Phe Gly Ile Leu His Ala
                   250                             260

ctc gct caa gcc ttc gtc act ggt ccc gcc acc aaa cgt ttc ggc gag   7888
Leu Ala Gln Ala Phe Val Thr Gly Pro Ala Thr Lys Arg Phe Gly Glu
                       270

aag cag gcc att atc gcc ggc atg gcg gcc gac gcg ctg ggc tac gtc   7936
Lys Gln Ala Ile Ile Ala Gly Met Ala Ala Asp Ala Leu Gly Tyr Val
           180                                 190

ttg ctg gcg ttc gcg acg cga ggc tgg atg gcc ttc ccc att atg att   7984
Leu Leu Ala Phe Ala Thr Arg Gly Trp Met Ala Phe Pro Ile Met Ile
                       200

ctt ctc gct tcc ggc ggc atc ggg atg ccc gcg ttg cag gcc atg ctg   8032
Leu Leu Ala Ser Gly Gly Ile Gly Met Pro Ala Leu Gln Ala Met Leu
    210                             220

tcc agg cag gta gat gac gac cat cag gga cag ctt caa gga tcg ctc   8080
Ser Arg Gln Val Asp Asp Asp His Gln Gly Gln Leu Gln Gly Ser Leu
                   230                             240

gcg gct ctt acc agc cta act tcg atc act gga ccg ctg atc gtc acg   8128
Ala Ala Leu Thr Ser Leu Thr Ser Ile Thr Gly Pro Leu Ile Val Thr
                       250

gcg att tat gcc gcc tcg gcg agc aca tgg aac ggg ttg gca tgg att   8176
Ala Ile Tyr Ala Ala Ser Ala Ser Thr Trp Asn Gly Leu Ala Trp Ile
           260                                 270

gta ggc gcc gcc cta tac ctt gtc tgc ctc ccc gcg ttg cgt cgc ggt   8224
Val Gly Ala Ala Leu Tyr Leu Val Cys Leu Pro Ala Leu Arg Arg Gly
                       280

gca tgg agc cgg gcc acc tcg acc tga atggaagccg gcggcacctc         8271
Ala Trp Ser Arg Ala Thr Ser Thr
    290

gctaacggat tcaccactcc aagaattgga gccaatcaat tcttgcggag aactgtgaat 8331

gcgcaaacca acccttggca gaacatatcc atcgcgtccg ccatctccag cagccgcacg 8391

cggcgcatct cggggtcgac tctagaggat ccccgcaacg ctgtcagcgc tttccagtta 8451
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aacggctcca acgtcgccat aggtaattcc tcgcccggcc atacgatcgg gcaggtgccg 8511

ttggctatcg ccgtcgcctg actcatcaca ctatcttccg ctgcatcgcg aagggttttg 8571

accacttctt ccatctctcc gtgcgccgga tgccatgctc acgtacgcgg cttatcagat 8631

agtcgggcag gccgtcgttc cagcccaatg aggggaagct ggcgtggagc gatgccagca 8691

cctgctcctc aacaccgtaa tggccggcgg cgaacaggca ttcggcggta agcgcttcca 8751

gcccctttaat catcacgctg cggcacatct tgatagccga cacgctgcca acgtggttac 8811

caccatagcg ggcgttacat ccaagcgtgg tgagtaattc agcaattgcc tctgcctgtg 8871

gtccccccgt caacagcggc gttcggagtg cccctggggg gaccggcgcc atcaccgcta 8931

catcgacata agcgccgggc ttaaagcatt tggcagcctg acgcttggtc tgcggggcga 8991

cggagttaag gtcaagaaaa tactgcgtgt cggtcatcag cggtgcagct tgtgaggcga 9051

catccagggc ggatcccgcg gtgacggtgg aaaatatgag ttcggcacct gtcaacgcgt 9111

cagccaggga gattgccgcc cgcacgcctc cccgatgcgc cttcgttatc atcgcatcgc 9171

gctcaggacc ttgcagcttg caatcccaga cggtgactgg gttcactttt gccagtgcat 9231

ccgcaagaat gccacctgct tcaccataac ctataaacgt tattgtcgtc ataacagctc 9291

cttacgcggc cacacgtcgg ccggaatgca aacgtcgccc gcgaacagaa gtcgcgccgt 9351

acgcagcaga ccgcagcctg ccaactgccc attatcatca agccggagcg ccacgctgaa 9411

ttgggtaccg agctccgaat tgggtaccga gctcgaatta attcgagctc ggtacccggg 9471

gatcnnnnnn nnnnnnnnnn nnnnnnnnnn nnnnnnnnnn nnnnnnnnnn nnnnnnnnnn 9531

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nnnnnnnnnn nnnnnnnnnn nnnnnnnnnn nnnnnnnnnn nnnnnnnnnn nnnnnnnnnn 10671

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nnnnnnnnnn nnnnnnnnnn nnnnnnnnnn nnnnnnnnnn nnnnnnnnnn nnnnnnnnnn 10851

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(54) Title: RECOMBINANT HOSTS SUITABLE FOR SIMULTANEOUS SACCHARIFICATION AND FERMENTATION

(57) Abstract: The invention provides recombinant host cells containing at least one heterologous polynucleotide encoding a polysaccharase under the transcriptional control of a surrogate promoter capable of increasing the expression of the polysaccharase. In addition, the invention further provides such hosts with genes encoding secretory protein/s to facilitate the secretion of the expressed polysaccharase. Preferred hosts of the invention are ethanologenic and capable of carrying out simultaneous saccharification fermentation resulting in the production of ethanol from complex cellulose substrates.

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A. CLASSIFICATION OF SUBJECT MATTER
IPC 7 C12N15/56 C12N1/21 C12N15/70 C12P7/06 C12P7/10
C12N9/24

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 7 C12N C12P

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)
EPO-Internal, BIOSIS, EMBL, STRAND, WPI Data, PAJ, CHEM ABS Data

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	PUGSLEY A P ET AL: "SECRETION OF THE CELL SURFACE LIPOPROTEIN PULLULANASE IN ESCHERICHIA-COLI COOPERATION OR COMPETITION BETWEEN THE SPECIFIC SECRETION PATHWAY AND THE LIPOPROTEIN SORTING PATHWAY" JOURNAL OF BIOLOGICAL CHEMISTRY, vol. 266, no. 21, 1991, pages 13640-13645, XP002163502 ISSN: 0021-9258 the whole document --- -/--	1-10, 14-16

[X] Further documents are listed in the continuation of box C. [ ] Patent family members are listed in annex.

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Date of the actual completion of the international search	Date of mailing of the international search report
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In. ational Application No
PCT/US 00/14773

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication,where appropriate, of the relevant passages	Relevant to claim No.
X	HE S Y ET AL: "CLONED ERWINIA-CHRYSANTHEMI OUT GENES ENABLE ESCHERICHIA-COLI TO SELECTIVELY SECRETE A DIVERSE FAMILY OF HETEROLOGOUS PROTEINS TO ITS MILIEU" PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCES OF THE UNITED STATES, vol. 88, no. 3, 1991, pages 1079-1083, XP002163503 1991 ISSN: 0027-8424 cited in the application the whole document ---	1-10, 14-16, 19-27, 33-41, 45-53
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X	DATABASE EMBL 'Online! Accession AF109242, 29 March 1999 (1999-03-29) ZHOU S ET AL: "Expression vector Zm006 endoglucanase CelZ gene, partial cds." XP002163506 100% identity in 450 BP (full length) overlap with SEQ ID NO 1 ---	20-32
X	INGRAM L O ET AL: "EXPRESSION OF DIFFERENT LEVELS OF ETHANOLOGENIC ENZYMES FROM ZYMOMONAS MOBILIS IN RECOMBINANT STRAINS OF ESCHERICHIA COLI" APPLIED AND ENVIRONMENTAL MICROBIOLOGY,US,WASHINGTON,DC, vol. 54, no. 2, 1 February 1988 (1988-02-01), pages 397-404, XP000106588 ISSN: 0099-2240 page 395, left-hand column, last paragraph -right-hand column, paragraph 1 ---	54
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INTERNATIONAL SEARCH REPORT

Int ational Application No
PCT/US 00/14773

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT		
Category °	Citation of document, with indication,where appropriate, of the relevant passages	Relevant to claim No.
A	WOOD B E ET AL: "Production of recombinant bacterial endoglucanase as a co-product with ethanol during fermentation using derivatives of Escherichia coli KO11." BIOTECHNOLOGY AND BIOENGINEERING, vol. 55, no. 3, 1997, pages 547-555, XP002163505 ISSN: 0006-3592 abstract	

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